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THE MAJOR HISTOCOMPATIBILITY (MH) GENES: SOMATIC MUTATION AND  
MATE CHOICE IN CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*)

by  
Leila Helou

A Thesis  
Submitted to the Faculty of Graduate Studies  
through Great Lakes Institute for Environmental Research (GLIER)  
in Partial Fulfillment of the Requirements for  
the Degree of Master of Science at the  
University of Windsor

Windsor, Ontario, Canada  
2010  
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MATE CHOICE IN CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*)

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## STATEMENT OF ORIGINALITY

Chapter 1 of this thesis incorporates the outcome of research undertaken in collaboration with D.D. Heath, and M. Docker; however, the analyses used are novel.

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## ABSTRACT

An analysis of a library consisting of major histocompatibility (MH) class I  $\alpha_1$  and class II  $\beta_1$  sequences for Chinook salmon (*Oncorhynchus tshawytscha*) identified mutation clusters or “hotspots” in the sequence, as well as identical mutations occurring independently. This suggests that the MH genes in Chinook salmon undergo somatic mutation. In sperm competition experiments jacks sired a higher proportion of eggs than hooknose males. Furthermore, an analysis of variance indicated that cryptic female choice had occurred. To further investigate this, I genotyped parents and offspring at the MH class I and class II peptide binding region to determine whether the differential fertilization success is driven by variation in genetic compatibility between the ova and sperm. I found that mate choice for the MH class I gene had not occurred; however, jack sperm that were more similar at the MH class II gene to the female had higher fertilization success.

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## TABLE OF ABBREVIATIONS

Average Amino Acid Dissimilarity Index	AADI
Basepairs	bp
Cryptic Female Choice	CFC
Major Histocompatibility Complex	MHC
Major Histocompatibility	MH
Peptide Binding Region	PBR
Polymerase Chain Reaction	PCR
Single Nucleotide Polymorphism	SNP
Yellow Island Aquaculture Ltd.	YIAL

## CHAPTER ONE

### GENERAL INTRODUCTION

#### **The major histocompatibility complex (MHC) and mate choice**

Major histocompatibility complex (MHC) molecules are cell-surface glycoproteins encoded by a large cluster of genes commonly known as the MHC (Lawlor et al., 1990). MHC molecules bind foreign and self peptides in specialized grooves and present them to T cells, thereby triggering the appropriate immune response (Nei and Hughes, 1991). The MHC genes code for two categories of MHC peptide-binding molecules: class I and class II. The MHC class I genes are expressed in all nucleated cells, and these molecules present peptides derived from intracellular pathogens, such as those derived from the degradation of viral proteins, to CD8<sup>+</sup> T cells (Nei and Hughes, 1991). MHC class II molecules are constitutively expressed on antigen-presenting cells of the immune system such as macrophages, B-cells, monocytes, and dendritic cells (Ting and Trowsdale, 2002). These molecules present peptides derived from extracellular pathogens that are internalized by phagocytosis or endocytosis, to CD4<sup>+</sup> T cells (Nei and Hughes 1991). Both MHC molecules are composed of immunoglobulin-like domains ( $\alpha_3$  and  $\beta_2$ -m in class I,  $\alpha_2$  and  $\beta_2$  in class II), which interact with T cells, peptide binding regions (PBR;  $\alpha_1$  and  $\alpha_2$  in class I,  $\alpha_1$  and  $\beta_1$  in class II), and a hydrophobic trans-membrane domain (Lawlor et al., 1990). The genes encoding the PBR are typically highly polymorphic, and characterized by numerous loci and a high degree of allelic diversity in most species (Nei and Hughes, 1991). Genetic variation at the PBR is beneficial since it is thought to enhance immunocompetence, as a diverse repertoire of MHC molecules would be able to recognize and present a wider range of pathogens. There are examples in nature of species lacking MHC polymorphism, and consequently having greater susceptibility to pathogens. For instance, the cheetah and the cottontop tamarin are species lacking MHC allelic diversity at all loci, and as a result are highly susceptible to certain pathogens (O'Brian et al., 1985; Watkins et al., 1988).

Teleost fish represent approximately half of the extant fish species, and 99.8% of the ray-finned fishes (actinopterygians) belong to the teleosts (Volff, 2005). Teleosts provide a unique opportunity for studying the evolution of the MHC genes because,

unlike mammals, the genes encoding the  $\alpha$  chains of MHC class I, and the  $\alpha$  and  $\beta$  chains of MHC class II molecules are not linked, thus allowing independent evolution to occur (Grimholt et al., 2002). Since teleost class I and class II genes are unlinked, and because they do not form a complex they are more correctly referred to as MH genes (Stet et al., 2002). Teleost MH genes are studied extensively in an attempt to pinpoint genes responsible for disease resistance in economically important fishes, and to determine how MH polymorphisms are maintained through evolutionary mechanisms. In order to explain the maintenance of MH polymorphism, researchers have developed two disease-based models: the *overdominant (heterozygote advantage) hypothesis*, and/or *negative frequency-dependant selection hypothesis*. The *overdominant hypothesis* states that heterozygous individuals have an immunological advantage as they can present a wider range of pathogen-derived antigens due to a larger number of different MH molecules; thus, selection for heterozygous individuals maintains MH polymorphism in a population (Langefors et al., 2001). There is evidence supporting this theory, for example, one study examined the resistance of winter-run Chinook salmon (*Oncorhynchus tshawytscha*) to infectious hematopoietic necrosis virus (IHNV), and found that MH heterozygotes had a higher survival than MH homozygotes (Arkush et al., 2002). The alternative mechanism is *frequency-dependent selection*, where rare alleles are more advantageous than common alleles because parasites would have developed resistance to the more commonly encountered molecule over time (Langefors et al., 1998). Selection for the rare allele would increase its frequency in a population, thereby maintaining MH polymorphism. Although balancing selection for particular alleles associated with disease resistance has been demonstrated in teleosts (Langefors, 1998), frequency-dependant selection is problematic to prove empirically as allele frequencies would need to be monitored over time to show cycling dynamics between alleles and parasites.

Sexual selection is an evolutionary mechanism which favours selection of traits valuable for increasing reproductive success, and can also function to maintain MHC polymorphism in a population. The process of MHC-dependant mate preference involves female choice for more genetically dissimilar males in an attempt to produce offspring with maximized immunocompetence (Penn and Potts, 1999). The hypothesized mechanism is that MHC associated molecules serve as olfactory cues, which females

utilize when choosing a mate. For instance, studies on mice have shown that females use urinary odour cues to choose mates, and prefer MHC-dissimilar males, thus contributing to diversity at the MHC in offspring (Potts et al., 1991; Roberts and Gosling, 2003). MH dependant mate choice has also been demonstrated for the stickleback *Gasterosteus aculeatus*, and contrary to findings that promote MH diversity, mate choice provided offspring with an optimal number of MH alleles (Aeschlimann et al., 2003). A recent study on Chinook salmon (*Oncorhynchus tshawytscha*) also reported surprising results, and in this study mate choice occurred for more genetically similar males, resulting in offspring with more similar MH alleles (Yeates, 2009).

Female mate choice is typically pre-copulatory, and exercised behaviourally by the female, which will usually choose a mate on the basis of material benefits or physical characteristics that are indicators of high genetic quality (Tregenza and Wedell, 2000). There is also evidence for mate choice occurring at the sperm-egg level, without the involvement of a female pre-copulatory behavioural choice. One example for a mechanism of post-copulatory mate choice at the sperm-egg level is demonstrated by the comb jelly *Beroë ovata*. In this species, multiple sperm fertilize the oocyte cytoplasm, and the female pronucleus inspects and evaluates each of the male pronuclei before choosing to fuse with one of them (Ziegler et al., 2005). In external fertilizers such as teleosts, there is evidence for sperm selection by eggs based on the MH genes (e.g. Yeates et al., 2009; Turner et al., 2009; Wedekind et al., 2001); however, the exact mechanism has yet to be determined.

### **Chinook salmon, *Oncorhynchus tshawytscha***

Chinook salmon are the largest of the Pacific salmon, and in North America their distribution ranges from the Ventura River in California to Point Hope, Alaska, as well as the Great Lakes (Myers et al., 1998; Kocik and Jones, 1999). Chinook salmon have also been introduced to other parts of the world including New Zealand, Chile, and Argentina (Ciancio et al., 2005). Chinook salmon are anadromous, meaning they live in fresh water as juveniles, migrate to sea water where they sexually mature, then return to fresh water to spawn (Quinn, 2005). Chinook salmon are also semelparous, meaning they die shortly after spawning (Quinn, 2005). Characterization of Chinook salmon life histories is often based on the timing of the downstream migration of juveniles, and the upstream

migration of adults; however, it is important to note that there is much variation in timing of migration, and length of residencies (Quinn, 2005). In general, Chinook salmon juveniles, which overwinter in fresh water and emigrate to salt water in early spring, are referred to as “stream-type” with adult runs occurring in spring and summer (Schaffter, 1980). “Ocean-type” salmon emigrate to salt water within the first 3 months of life, and predominately make up the fall-run and summer-run populations (Reimers, 1973). Also, there is variation in age and size at reproductive maturity in male Chinook salmon. At the spawning site, male Chinook salmon compete for access to females’ eggs aggressively (hooknose) or through a sneaking tactic (jack). Which male phenotype a fish adopts is genetically and environmentally determined, and the incidence of jacking varies from 10% to over 90% in some populations (Healey, 1991; Heath et al., 1994; Heath et al., 1991).

The impact of salmon fisheries as well as environment degradation has led to a decline in natural spawning populations of Pacific salmon to the extent that several populations have been placed under the U.S. Endangered Species Act. As a result, a large number of Pacific Northwest hatcheries have been propagating fish in order to supplement wild populations. For instance, over 200 million juvenile anadromous Pacific salmon are released into the Columbia River annually, and hatchery fish represent almost 85% of returning salmonids (Campton, 2004). Whether salmon hatcheries are augmenting wild populations successfully is controversial, and commonly used fertilization protocols have been criticized for causing artificial selection and inbreeding (Campton, 2004). Thus, the aim of several hatcheries has been to employ breeding methods to minimize genetic change as well as maintain genetic diversity to ensure the program’s success.

Due to the commercial importance of salmon, the MH genes of salmonids are well characterized and available for 16 different species (Wegner, 2008). Although salmonids underwent an entire genome duplication approximately 100 million years ago, they have very few MH loci in comparison to other fish species (Miller et al., 2002). For instance, Atlantic cod may have as many as 42 MH class I loci (Miller et al., 2002), while only three class I loci in Pacific (*Oncorhynchus* spp.) and Atlantic salmon (*Salmo salar*) have been identified: A, B, and UA, and none are the result of the gene duplication

(Grimholt et al., 1993; Miller and Withler, 1998). Furthermore, failure to isolate cDNA from the B locus, and its substantial divergence from the other loci has led researchers to believe that the B locus is a pseudogene (Grimholt et al., 1993; Miller and Withler, 1998). In addition, only one MH class II gene has been identified in salmonids, and it is substantially less polymorphic than class I genes (Miller and Withler, 1996).

### **Somatic mutation**

Somatic mutation has been extensively studied in the variable region of immunoglobulins (Ig) as it is believed to be the mechanism for the generation of new antibodies during the primary immune response (Yang et al., 2006). In this process, mutated B cells expressing higher affinity antibodies on the cell surface are selected for by antigens, while B cells expressing lower affinity receptors presumably undergo apoptosis (Han et al., 1995). Several features of the somatic mutation mechanism in Ig has been described (reviewed in Denépoux et al., 1997), yet the possibility of somatic mutations occurring in the peptide binding region of the MHC has not yet been proposed or investigated.

The mechanism for the generation of novel MHC alleles is yet to be proven; however, research shows that recombination and gene conversion may be important processes for the generation of MHC allelic diversity (Martinson et al., 1999). Alternately, the MH genes may be diversifying through point mutations occurring in somatic cells. Differences in the MH alleles of Chinook salmon are produced by relatively small differences in large numbers of nucleotides. For example, an analysis of MH class II  $\beta_1$  gene in Chinook salmon revealed three alleles differing only in 3-7 nucleotides; therefore, point mutations rather than recombination or gene conversion, is more likely the mechanism for diversifying alleles at the MH genes (Miller et al., 1997). For species that lack MH diversity, such as Chinook salmon, somatic point mutation would be an important mechanism for the creation of new MH alleles and would explain how the fish maintain immunological competence in two very different aquatic environments (fresh and salt water) despite having a limited number of functional MH loci. It is important to note that in order for the new alleles to be heritable, mutation would have to occur in the germ cells.

## STUDY OBJECTIVES

**Chapter two** analyzes a large MH class I and II sequence library in order to quantify and characterize base pair substitutions, and investigates the effects of PCR conditions on the frequency of such mutations. In the MH sequence library, regions of the MH gene containing a high number of mutations were investigated in order to determine whether mutations were occurring in clusters at “hotspots” or spread randomly along the gene. A series of PCR-based experiments were conducted to determine how polymerase type and the number PCR cycles affect the frequency of mutations observed. From these analyses I provide valuable insight into the nature of PCR induced MH artifacts versus genuine somatic mutations.

**Chapter three** investigates the outcome of sperm competition between jack and hooknose Chinook salmon and the role of MH variation in that process. An artificial breeding experiment was performed to produce 25 full- and half-sib families with jack and hooknose male parent sperm competing for fertilization, and microsatellite analysis was used to determine parentage of offspring. From the microsatellite analysis I determine which male (jack versus hooknose) sired the majority of offspring, and whether there are any significant sire, dam, and sire-dam interaction effects. In Chapter one I used cloning and sequencing to accurately genotype parents and offspring for the MH gene in order to determine whether the outcome of sperm competition was associated with the MH genotype in parents. From this I was able to investigate whether female choice is occurring for sperm expressing more similar/dissimilar MH alleles.

Results from chapter two and three help us understand the mechanisms for MH evolution, and the source of MH polymorphism in Chinook salmon, allowing us to gain insight on key evolutionary processes, and enabling us to utilize this information to develop successful breeding protocols in hatcheries.



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## CHAPTER TWO

Mutation analysis of the Major Histocompatibility (MH) genes in Chinook salmon  
(*Oncorhynchus tshawytscha*)

## INTRODUCTION

The major histocompatibility complex (MHC) includes genes of special interest in evolutionary biology due to their roles in disease resistance, mate choice, and kin recognition (Bernatchez and Landry, 2003). The MHC genes code for membrane-spanning glycoprotein heterodimers that bind both self and foreign antigens. At the cell surface, the MHC-antigen complex interacts with T cells, which recognize an antigen only when combined with an MHC molecule (Cuesta et al., 2005). There are two types of MHC peptide-binding molecules (class I and class II) differing in structure and function: In the cell, foreign, endogenously synthesized proteins, such as those derived from a virus, are degraded within the cytoplasm, and are presented by MHC class I molecules to CD8<sup>+</sup> T cells (Cuesta et al., 2005). Pathogens and proteins that are exogenously derived are internalized within the cell by phagocytosis or endocytosis, and are presented by MHC class II molecules to CD4<sup>+</sup> T cells (Cuesta et al., 2005). Both types of MHC molecules are composed of immunoglobulin-like domains (which interact with T cells), peptide binding regions (PBR), and a hydrophobic trans-membrane domain (Lawlor et al., 1990). The highest level of polymorphism observed in the MHC genes is concentrated within the sequences encoding the PBR, and this variability is correlated to breadth of pathogen recognition and disease resistance (Klein and Figueroa, 1986). Although the class I and class II genes form a linkage group in mammals, the genes encoding the  $\alpha$  chains of MHC class I and the  $\alpha$  and  $\beta$  chains of MHC class II molecules are not linked in teleost fishes; as they do not form a single complex, they are more correctly referred to as MH genes in teleosts (Stet et al., 2002). Furthermore, since the class I and class II loci are not physically linked in teleosts, independent evolution can occur, making the MH

genes in teleosts a good model for molecular evolution studies (Grimholt et al., 2002).

The degree of polymorphism in the MHC genes varies greatly by species and by population within species (Miller et al., 1997). For instance, the MHC in humans is estimated to contain 421 loci; whereas, salmonids are estimated to have only 3 class I genes (designated A, UA, and B), and only 1 class II gene (Horton et al., 2004; Miller and Withler, 1998). Furthermore, the MH class I B locus in salmonids is likely a non-classical MH locus, due to the failure to isolate cDNA, and its reduced  $\alpha 2$  variability compared to those of MH class I A (Grimholt et al., 1994). Salmonids underwent a whole-genome duplication 25-100 million years ago resulting in the current pseudotetraploidy state, yet this event was not followed by a corresponding increase in the number of functional MH loci (Allendorf and Thorgaard, 1984).

MHC polymorphism can be attributed to a variety of mutagenic mechanisms such as gene duplications, deletions, point mutations, recombination, and gene conversion; however, such novel mutations must occur in the germ-line for selection to act on them (Lawlor et al., 1990). A single nucleotide substitution resulting in an amino acid change in the PBR sequences can create a new/rare allele, having important fitness consequences for the organism (Lanfords et al., 1998). Miller et al. (1997) examined MH evolution in Chinook salmon (*Oncorhynchus tshawytscha*) and found that much of the variability in MH class I  $\alpha 1$  alleles were produced by point mutations, as well as inter-allelic exchange or conversion. Furthermore, Miller et al. (1997) discovered that most of the point mutations found in the PBR genes were nonsynonymous indicating that selection is responsible for maintaining the polymorphisms.

The MH genes have been used widely for behavioural, population genetic, and

survival-related evolutionary analyses in salmon (e.g. Landry et al., 2001; Evans et al., 2009; Pitcher and Neff, 2006). The likelihood of MH genotyping error is high, yet only one systematic assessment of MH genotyping error or mutation in teleosts has been published (Lenz and Becker, 2008). *In vitro* PCR conditions are known to significantly influence the mutation rate, and numerous methods have been proposed to eliminate artificial amplicons during and after the PCR (Borriello and Krauter, 1990; Eckert and Kunkel, 1991; Zylstra et al., 1998; Thompson et al., 2005). Several population genetic studies have discovered singleton mutants in individuals, and dismissed these as likely PCR artefacts (Alcaide et al., 2008; Grimholt et al., 2002; Grimholt et al., 1994). Alternatively, somatic hypermutation occurring *in vivo*, an important mechanism for maintaining variation in immune genes, may be responsible for some of those single-copy mutants. Due to the relatively low number of functional MH loci in salmonids, it would be beneficial for the salmon to have alternative mechanisms of allele diversification such as somatic mutation. Somatic hypermutation is seen in the variable regions of the immunoglobulin (Ig) genes (Lee et al., 2002; Yang et al., 2005); however, there have been no studies to date on the potential for such mutations to occur in the peptide binding region of the MH genes.

In this study we conduct a large-scale survey of rare single-copy MH PBR sequences in 67 Chinook salmon and report a very high frequency of such alleles. Detailed characterization of those mutations identified potential somatic mutations as well as mutation hotspots in the MH class I and II PBR. We also use a series of PCR-based experiments to investigate how PCR conditions affect mutation frequencies, and show that the polymerase used can significantly increase base pair substitutions (BPS)

and chimera mutations and increasing the number of PCR cycles increases chimera mutations. This analysis, coupled with the sequence survey, provides insight on the relative occurrence of PCR artefacts and somatic mutations in Chinook salmon.

## METHODS AND MATERIALS

Our analyses consisted of two separate approaches: 1) a detailed analysis of an MH sequence library consisting of 396 MH class I  $\alpha 1$  sequences (n=38 fish), and 389 MH class II  $\beta_1$  sequences (n= 29 fish); and 2) an experimental evaluation of the contribution of PCR artefacts to the observed frequency of rare MH sequence mutants.

### **Sequence Library**

Two sequence libraries were used to characterize and quantify mutations occurring in Chinook salmon MH genes across multiple individuals. The fish were all from Yellow Island Aquaculture in British Columbia, and DNA was extracted either from fin tissue or blood using a standard plate-based extraction method (Ephinstone et al. 2003). The MH class I  $\alpha 1$  sequence library (396 sequences) was constructed using primers previously developed (Miller et al. 1997). The sequence of the sense primer was 5'-TGA CTC ACG CCC TGA AGT A-3', and the anti-sense primer was 5'-CTC CAC TTT GGT TAA AAC G-3' producing either a 228bp and/or a 222bp fragment(s). The PCR consisted of: 1 $\mu$ L of extracted DNA, 0.5  $\mu$ L of each primer (100ng/ $\mu$ L), 2.5  $\mu$ L 10X PCR buffer (10mM Tris-HCl (pH-8.4) 50mM KCl), 3.0 $\mu$ L of MgCl<sub>2</sub> (25mM), 1.0 $\mu$ L of dNTP's (200 $\mu$ M), and 0.5 units of DNA *Taq* polymerase (Invitrogen), and ddH<sub>2</sub>O to make a 25 $\mu$ L reaction. The reaction profile consisted of: 2 min initial denaturation (95°C), 30 cycles of 30 sec denaturation (95°C), 30 sec annealing (52°C), 1 min extension (72°C), and 10 min extension (72°C). The MH class II  $\beta_1$  sequence library (389



sequences) was constructed using primers previously developed by Docker and Heath (2002), and conditions identical to those used to amplify the  $\alpha_1$  gene. The sequence of the sense primer was 5'-CCG ATA CTC CTC AAA GGA CCT GCA-3', and the anti-sense primer was 5'-GGT CTT GAC TTG MTC AGT CA-3' producing a 294bp fragment. PCR products were ligated into a TA cloning vector, and inserts were amplified using the M13 forward primer (5'-GTA AAA CGA CGG CCA GT-3') and M13 reverse primer (5'-AAA CAG CTA TGA CCA TG-3'). The reactions were comprised of: 50-100 ng of template, 0.5  $\mu$ L of each primer, 2.5  $\mu$ L of 10X PCR buffer, 2.5 mM  $MgCl_2$ , 200  $\mu$ M dNTPs, 0.5 units DNA *Taq* polymerase (Sigma), and ddH<sub>2</sub>O to adjust final volume to 25  $\mu$ L. On average 10 sub-clones were used for sequencing for each fish. Inserts were sequenced using a 1/8<sup>th</sup> reaction of a CEQ DTCS Quick Start Kit and analyzed on a CEQ 800 automated DNA sequencer (Beckman Coulter, Fullerton, CA). Sequence variants were confirmed in 15 sub-clones by reverse sequencing; therefore, we rejected sequencing error as the source of the sequence variation.

### **Analysis of Sequence Library**

Alignments were constructed for all the MH class I  $\alpha_1$  sequences, and all class II  $\beta_1$  sequences separately using Geneious Pro (Biomatters Limited) software. Because fish samples originated from a single captive population, there were relatively few alleles, and their identification was possible even with as few as 4 clones per fish. Once alleles were identified, base pair substitutions were recorded, and mutation frequency was calculated for each gene. To allow visualization of the relationships between the sequence variants, TCS version 1.21 was used to create a haplotype network with a subset of alleles and mutants obtained from the  $\alpha_1$  gene (Clement et al., 2000). To identify alleles versus

mutant sequences, a frequency distribution histogram was created for the MH class I  $\alpha_1$  sequences, and alleles were identified by a sequence frequency greater than four. The MH class II  $\beta_1$  gene is substantially less polymorphic and detection of alleles was made directly by viewing alignments, and determining which sequences had occurred at a high frequency.

The number of nonsynonymous and synonymous base pair substitutions was determined for both  $\alpha_1$  and  $\beta_1$  alleles and base pair substitution (BPS) mutants. Cross tabulation was used to determine whether the number of nonsynonymous and synonymous substitutions in the true alleles differed significantly from the substitutions found in the BPS mutants.

Chimera mutants were identified as having sequence polymorphisms from the two recognized alleles recombined to generate an apparently novel allele sequence. Chimera mutations were excluded from the mutation rate calculation, as studies have suggested these are likely PCR artefacts (Judo et al., 1998; Lenz and Becker, 2008; see below).

A 20-base-pair sliding window analysis was used to determine whether specific sequence regions had higher (or lower) substitution rates than expected. For each 20-base-sliding window, the number of mutations was counted and an average across all sliding windows was calculated. Mutation rate estimates obtained from individual sliding windows were used in a Z-score test to determine the probability of observing that value by chance.

### **PCR Treatments**

To investigate the effect of PCR conditions on mutation frequency in the MH II  $\beta_1$  gene, we ran a series of PCR treatments using DNA from two fish (Table 1). Each

Table 1. The experimental design used to determine the effect of PCR conditions on mutation frequency in the MH class II  $\beta_1$  region. AmpliTaq<sup>®</sup> polymerase and Phusion<sup>®</sup> polymerase were used to PCR amplify the MH class II  $\beta_1$  region in Chinook salmon. All treatments include the 30 PCR cycles conducted after cloning in the total number of PCR cycles.

Treatment	DNA polymerase	Total No. of PCR cycles
1	AmpliTaq <sup>®</sup>	60
2	AmpliTaq <sup>®</sup>	90
3	AmpliTaq <sup>®</sup>	120
4	AmpliTaq <sup>®</sup>	150
5	Phusion <sup>®</sup>	60
6	Phusion <sup>®</sup>	90
7	Phusion <sup>®</sup>	120
8	Phusion <sup>®</sup>	150

treatment differed in the number of total PCR amplification cycles (60, 90, 120, and 150 cycles), and each reaction was conducted with either AmpliTaq<sup>®</sup> (Applied Biosystems), or Phusion<sup>®</sup> High Fidelity polymerase (Finnzymes). Phusion<sup>®</sup>, unlike AmpliTaq<sup>®</sup>, has proof-reading ability, and is a *Pyrococcus* (*Pfu*)-like enzyme with a double-stranded DNA binding domain, which reportedly increases processivity. The expectation is that the observed frequency of mutations should be greater in the treatments with AmpliTaq<sup>®</sup> polymerase and with greater number of PCR cycles, if the mutations are resulting from PCR error. Furthermore, mutations should occur randomly across the PBR sequence if they are indeed artefacts, so identical mutations should not be observed in separate reactions. The conserved MH class II  $\beta_2$  region was used as a control, since it was found to be highly conserved in comparison to the PBR region (Miller and Withler, 1996). If most of the mutations were generated by PCR error, we would predict similar mutation rates in both  $\beta_1$  and  $\beta_2$  regions.

### **DNA extraction and amplification**

Chinook salmon collected from Yellow Island Aquaculture Ltd. (YIAL) in British Columbia, Canada were used for this experiment. DNA was extracted from fin tissue using a standard plate based extraction method (Elphinstone et al., 2003). The PBR  $\beta_1$  of MH class II was PCR amplified with primers developed by Docker and Heath (2002) to produce a 294 bp fragment. The PCR conditions used are identical to those used for the sequence library except 1.0 units of AmpliTaq<sup>®</sup> polymerase (Applied Biosystems) was used. For treatments consisting of reactions greater than 60 total PCR cycles, 1  $\mu$ L of PCR product (obtained using the previous PCR) was added to a fresh master mix, and run for an additional 30 cycles. This was repeated until the number of desired PCR cycles was

achieved. For PCRs utilizing Phusion<sup>®</sup>, the reagent concentrations consisted of 1 $\mu$ L of extracted DNA, 0.7 $\mu$ L of each primer (100ng/ $\mu$ L), 4 $\mu$ L of 5x Phusion<sup>®</sup> HF buffer, 0.4 $\mu$ L of dNTPs (200 $\mu$ M), 0.2 $\mu$ L (0.02U/ $\mu$ L) of Phusion<sup>®</sup> DNA polymerase (Finnzymes), and ddH<sub>2</sub>O to make a 20 $\mu$ L reaction. The thermal cycler profiles used were identical to those used to amplify the  $\alpha_1$  and  $\beta_1$  gene, described above.

The conserved  $\beta_2$  region (control) was PCR amplified in two fish using AmpliTaq<sup>®</sup> polymerase for 120 cycles. The sense primer (5'-CCG ATA CTC CTC AAA GGA CCT GCA-3'), is situated at the N-terminal region of the  $\beta_1$  domain (Miller and Withler, 1996), and the antisense primer (5'-GGT CTT GAC TTG MTC AGT CA-3') is located at the end of the  $\beta_2$  domain. The PCR conditions used were the same as those used to amplify the  $\beta_1$  domain. These primers amplified a single fragment approximately 290 bp in length.

Following PCR amplification, the products were cloned into the pGEM<sup>®</sup>-T vector (Promega) following the manufacturer's protocol. The insert was amplified using the M13 forward and reverse primers under the following conditions: 2 min initial denaturation (94 $^{\circ}$ ); then 35 cycles of 1 min denaturation (94 $^{\circ}$ ), 1 min annealing (55 $^{\circ}$ ), 1 min extension (72 $^{\circ}$ ); a final 3 min extension cycle (72 $^{\circ}$ ). Each 25 $\mu$ L reaction consisted of: 50-100ng of template DNA, 0.05 $\mu$ g of each primer, 0.5 units of DNA Taq polymerase, 25 mM MgCl<sub>2</sub>, and 200  $\mu$ M dNTPs. Approximately 29-65 sub-clones containing inserts were cleaned for each PCR-cycle treatment using AMPure (Agencourt) purification system, and sequenced using the M13 forward primer along with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). After purification using CLEANseq (Agencourt), sequencing was performed using ABI BigDye Terminator version 3.1 on an

ABI 3130xl sequencer.

### **Data Analysis**

Alignments were constructed for all  $\beta_1$  sub-clone sequences (N=397), and  $\beta_2$  sequences (N=58) with Geneious Pro (Biomatters Limited) software. Alleles were identified as the sequences occurring at the greatest frequency, and remaining sequences were characterized as either chimera or BPS mutants. Chimera and BPS mutation frequency was determined separately for each treatment by counting the number of sequences containing the mutation divided by the total number of sequences in that treatment. Mutations were pooled across all treatments for each polymerase type, and cross tabulation was used to determine whether there were significant differences between the total number of chimera and BPS mutants using AmpliTaq<sup>®</sup> versus Phusion<sup>®</sup>. A log-linear analysis was conducted to determine whether there was a significant effect of polymerase type on the frequency of BPS and chimeras produced, and whether the number of PCR amplification cycles (when combining all treatments) had a significant effect on the frequency of BPS and chimeras produced (SYSTAT version 12). Finally, cross-tabulation was used to determine whether the number of mutations observed in the  $\beta_2$  exon (n= 58) was significantly different from the mutation count observed in the  $\beta_1$  exon.

## **RESULTS**

### **Sequence Library**

**MH class I  $\alpha_1$ :** Analysis of 396 MH class I  $\alpha_1$  sequences (n=38 fish) revealed 86 distinct sequences. Fifteen alleles were identified, with seven of those previously identified (GenBank accession numbers: DQ647923, DQ647922, U80293, U80273,

U80284, AF362114, and AF162868). BPS in the MH class I  $\alpha_1$  sequences occurred at a frequency of 15.2%, and chimeras occurred at a frequency of 4.8%. For this study we further analyze BPS, but excluded chimeras from our analyses since they are known to be common PCR artefacts. Sequences with only one BPS represented 88.3% of the BPS mutants, and sequences having two BPS represented 11.7% of the BPS mutations. The haplotype network for alleles DQ647922 and U80284 shows a typical BPS mutation pattern dominated by single BPS mutations (Figure 1a). Of the sequences with mutations a large proportion of the sequences 72% were singletons i.e. were found only once in the sequence library (Figure 1b). Across all sequences and both genes, the majority of BPS (88.1%) occurred in only a single clone sequence (Figure 2a). Identical BPS occurring twice (in separate PCRs) represented 6.8% of mutations, and identical BPS occurring three times represented 5.1% of mutations (Figure 2a). As expected, most of the BPS in both the true MH PBR  $\alpha_1$  alleles and the mutations were nonsynonymous (87% and 60% respectively). However, the frequency of nonsynonymous substitutions in the  $\alpha_1$  alleles was significantly greater than in the BPS mutations ( $\chi^2=14.83$ ;  $p<0.0010$ ).

MH class I  $\alpha_1$  transitions made up 75% of the observed BPS, while transversions represented 25% of mutations. Furthermore, A→G mutations occurred at the highest frequency (33.3%; Table 2), followed by T→C mutations (20%; Table 2). The frequency of BPS mutations found in the 37-61bp (z score=2.39;  $p=0.0082$  and z score=1.88;  $p=0.03$ ), and 113-132bp (z score=1.88;  $p=0.03$ ) region of the  $\alpha_1$  gene was found to be significantly higher than background (Figure 3a). Also, in the MH class I  $\alpha_1$  gene the 154-194bp region had significantly lower levels of base-pair substitution than background (z score = -2.25;  $p=0.012$  and z score=-1.73;  $p=0.04$ ; Figure 3a).

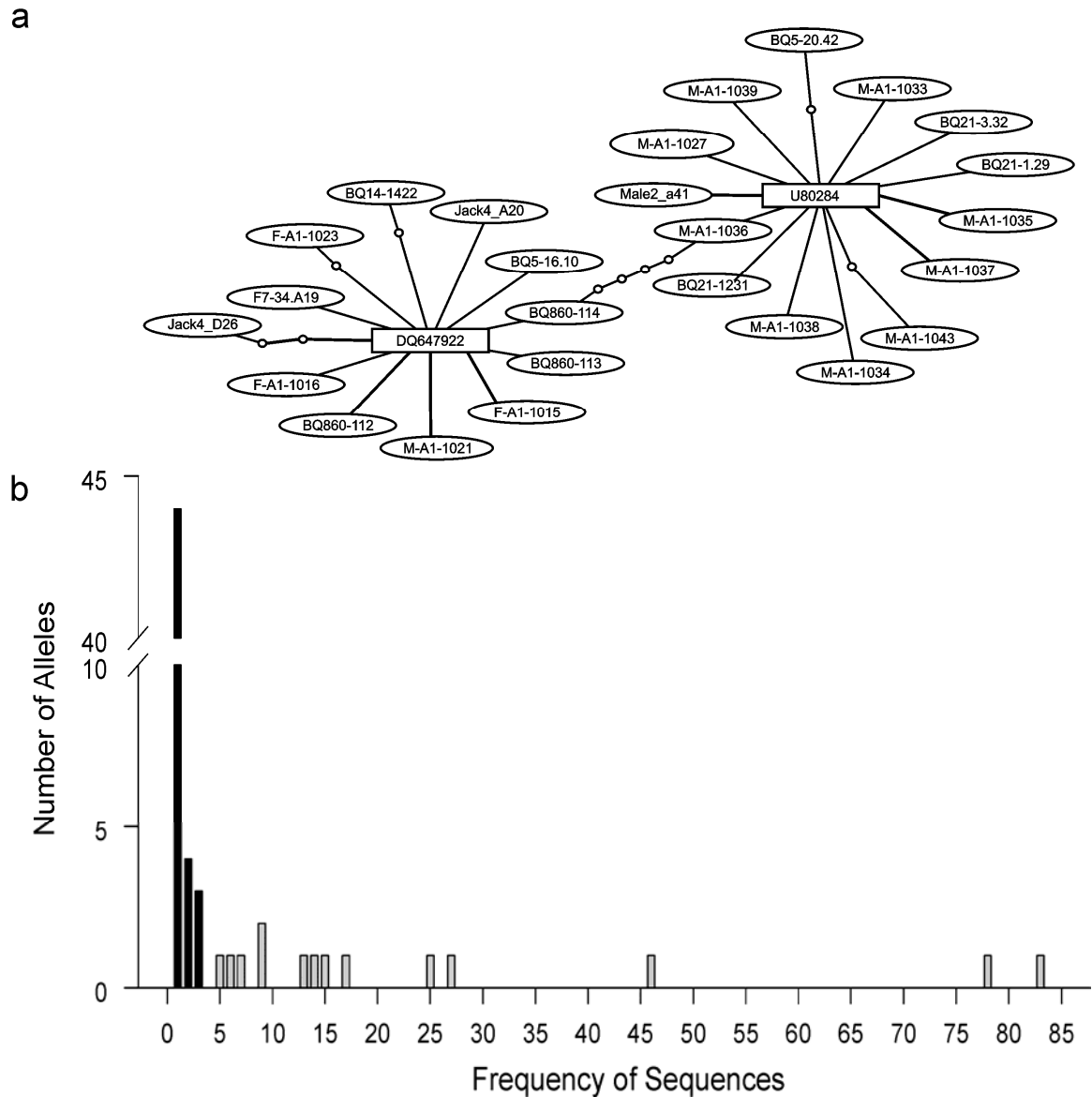


Figure 1. Panel a: Haplotype network of a subset of sequences ( $n=31$ ) obtained from the MH class I  $\alpha_1$  gene library for Chinook salmon. Allele labels are enclosed by rectangles and mutants by ovals. The number of connecting branches represents the number of base pair differences between sequences. Panel b: The number of alleles and their respective sequence frequencies for the MH class I  $\alpha_1$  gene library ( $N=396$ ) for Chinook salmon. Sequence frequencies greater than five (in grey) are the alleles.



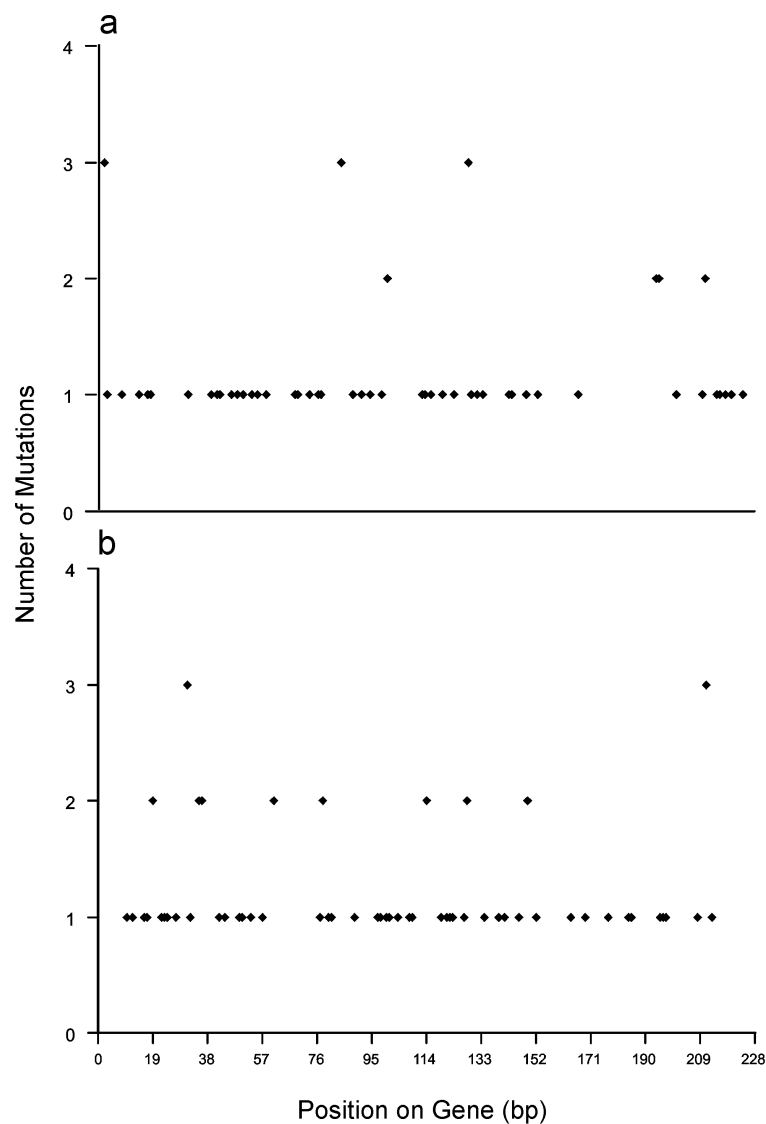


Figure 2. The position and frequency of single base pair substitutions (BPS) along the gene in the 3' to 5' direction (complementary to the sense primer) resulting from separate PCR reactions. Panel a: MH class I  $\alpha_1$  peptide binding region (N=61 BPS; 222-228 bp), and Panel b: MH class II  $\beta_1$  peptide binding region (N=68 BPS; 213 bp).

Table 2. Distribution of nucleotide substitutions in the MH class I  $\alpha_1$  (n=61 mutations) and MH class II  $\beta_1$  (n=68 mutations) sequence libraries from Chinook salmon. Bolded frequencies represent the two highest values.

		Original Base					
		A	T	G	C	Total	
Substitution	MH class I $\alpha_1$	A		0.050	0.100	0.017	0.230
		T	0.067		0.050	0.117	0.475
		G	<b>0.333</b>	0.000		0.017	0.131
		C	0.050	<b>0.200</b>	0.000		0.164
		Total	0.450	0.250	0.150	0.150	1.000
	MH class II $\beta_1$	A		0.015	0.044	0.000	0.059
		T	0.029		0.044	0.162	0.235
		G	<b>0.412</b>	0.000		0.000	0.412
		C	0.029	<b>0.265</b>	0.000		0.294
		Total	0.471	0.279	0.088	0.162	1.000

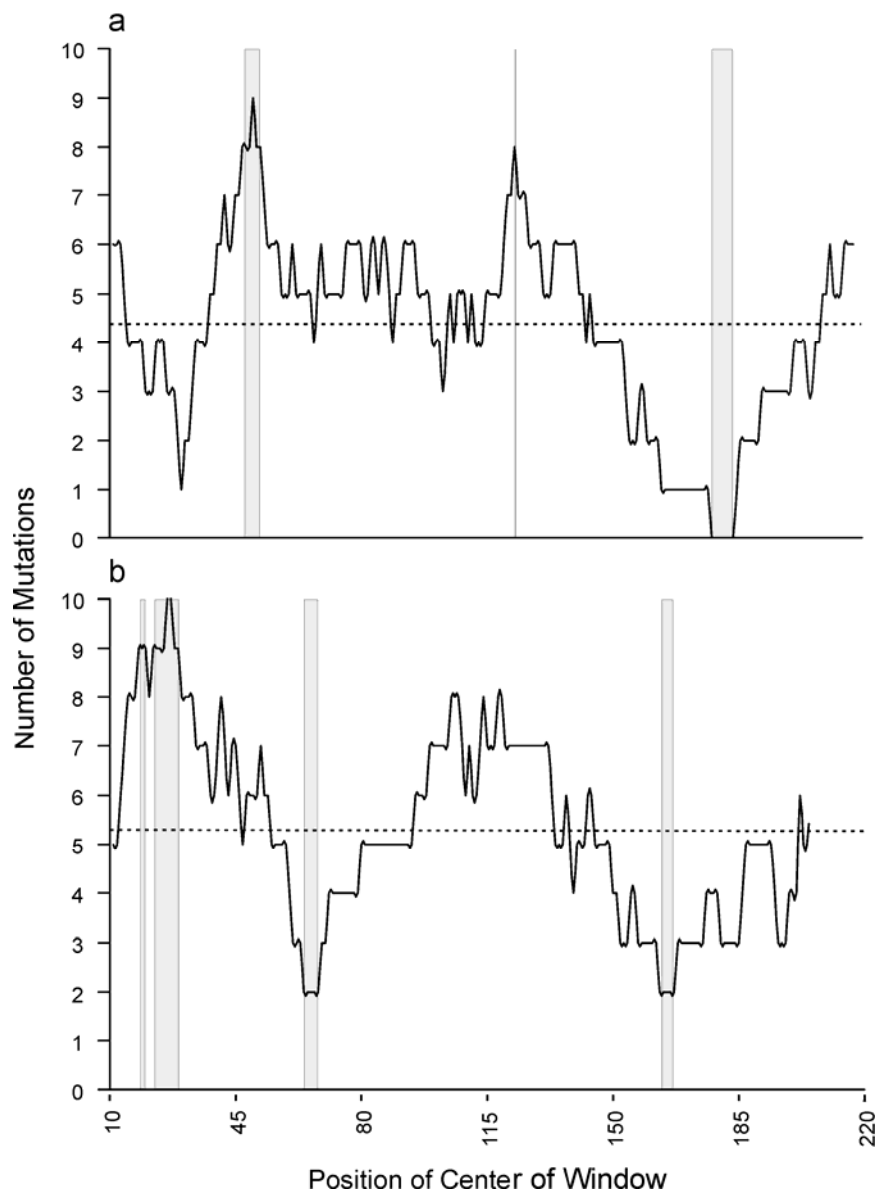


Figure 3. Line graphs showing the number of mutant base pairs in a sliding 20-base-pair window analysis of the MH sequence library data. Panel a: MH class I  $\alpha_1$  PBR, and Panel b: MH class II  $\beta_1$  PBR. The dashed lines indicate the mean mutation value across the entire region, and grey shaded areas represent mutation counts in the sliding window found outside the 95% confidence interval.

**MH class II  $\beta_1$ :** The 389 MH class II  $\beta_1$  sequence library (n= 29 fish) revealed 73 distinct sequences. Four alleles were identified in our MH class II  $\beta_1$  sequence library and all have been reported previously (GenBank accession numbers: DQ450874, AY100006, AY100007, and AY100008). The BPS frequency in the MH class II  $\beta_1$  sequence library was 17.5%, and the frequency of chimeras was 3.3%. Sequences with only one BPS accounted for 82.9% of the total identified mutations; two BPS sequences represented 15.8% of mutations, and one sequence was found to have 3 BPS representing 1.3% of mutations. Most mutations (84%; Figure 2b) were unique, occurring only once in the sequence library; however, 13% of mutations (Figure 2b) were found occurring in another sequence generated from a separate PCR reaction, and two mutations occurred in three separate reactions (3%; Figure 2b). Similar to the  $\alpha_1$  gene, the number of nonsynonymous and synonymous substitutions in  $\beta_1$  alleles differed significantly from the number of substitutions in the sequences with BPS mutations ( $\chi^2=5.59$ ;  $p=0.018$ ) with the alleles having a larger proportion of nonsynonymous mutations than the mutants (88.8% and 74% respectively).

Analysis of nucleotide substitutions at MH class II  $\beta_1$  revealed that transitions occurred at a frequency of 88%, with transversions occurring at a frequency of 13%. There was a strong bias for A→G substitutions (41.2%), followed by T→C substitutions (26.5%; Table 2). The 20 base sliding window analysis revealed that the 17-36bp region had a significantly elevated frequency of BPS ( $\chi^2=5.062$ ;  $p=0.024$ ) (Figure 3b).

#### **Analysis of PCR Effects on Mutations**

The possibility of somatic mutations in the MH PBR has been masked by a perception of high levels of PCR error, and researchers consequently dismissed all “rare”

MH PBR sequences as probable PCR artefacts. Thus the purpose of our PCR experiments was to determine the effect of PCR conditions on MH PBR mutations, as well as to characterize those mutations. The number of clones sequenced for each treatment ranged from 29 to 65, giving a total of 397 sequences from two fish for the MH class II  $\beta_1$  gene: two alleles were identified differing by six base pair substitutions. The first allele (GenBank accession: AY100008) represented 5.4% of the sequences, the second allele (GenBank accession: DQ450874) represented 53.0% of the sequences, and the remaining sequences exhibited mutations and were placed in either the BPS or chimera category. Of the mutant sequences, three were found in GenBank (accessions AY100009, EF432121, and EF432117), and all were chimeras.

Of the mutations produced with AmpliTaq<sup>®</sup> polymerase, 50% were chimeras, while the remaining mutant sequences were BPS. Of the mutations produced by Phusion<sup>®</sup> polymerase, 94% were chimeras, while only 6% were BPS. Pooling sequences across all treatments, there is a significant difference ( $\chi^2=39.96$ ;  $p<0.001$ ) in the total number of chimeras produced by the two polymerases, with Phusion<sup>®</sup> producing a greater frequency (41.0%) than AmpliTaq<sup>®</sup> (13.1%). A log-linear analysis used to test for the effect of polymerase and PCR cycle number revealed polymerase type significantly affected chimera production ( $\chi^2=23.15$ ;  $p<0.001$ ), as did the number of PCR amplification cycles ( $\chi^2=73.13$ ;  $p<0.001$ ; Figure 4). AmpliTaq<sup>®</sup> produced a greater frequency of BPS (13.1%) than Phusion<sup>®</sup> polymerase (2.7%), and the effect of polymerase type was significant ( $\chi^2=16.16$ ;  $p<0.001$ ). The number of PCR amplification cycles did not have a significant effect on the frequency of BPS across all treatments ( $\chi^2=2.731$ ;  $p=0.44$ ).

An alignment of all of the MH class II  $\beta_2$  (trans-membrane) sequences (N = 58)

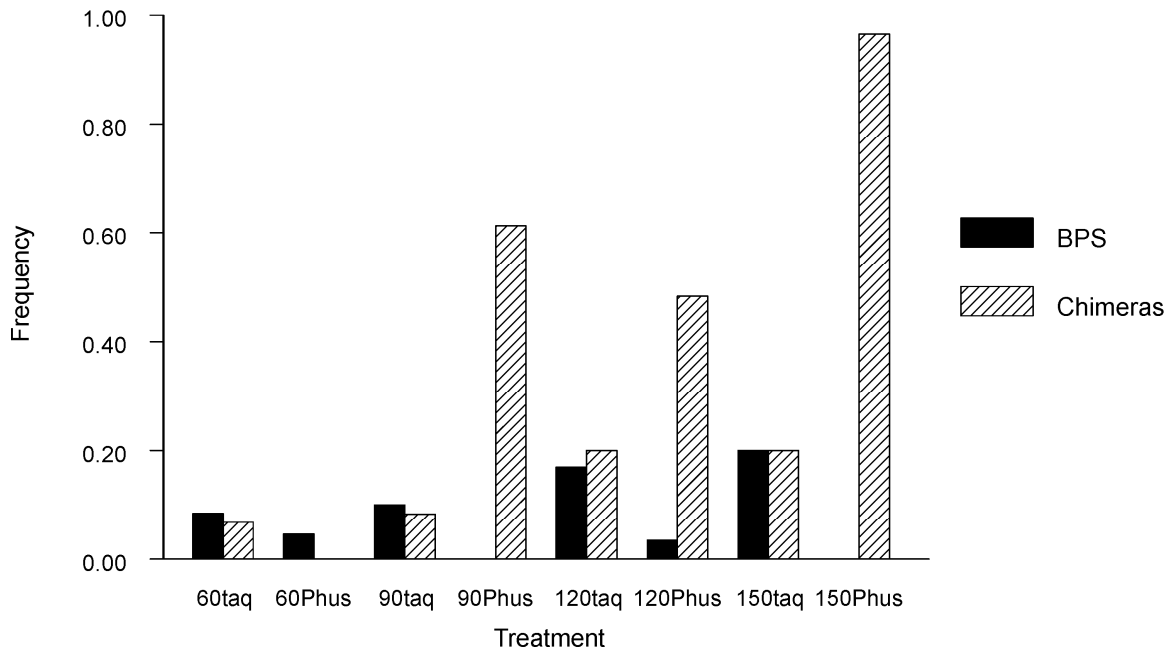


Figure 4. The frequency of chimeras and base-pair substitutions (BPS) with various PCR cycle numbers and two polymerase enzymes AmpliTaq® and Phusion®. The number of PCR amplification cycles significantly affected chimera frequency ( $\chi^2=73.13$ ;  $p<0.001$ ), as did polymerase type ( $\chi^2=23.15$ ;  $p<0.001$ ). Polymerase type had a significant effect on BPS frequency ( $\chi^2=16.16$ ;  $p<0.001$ ); however, the number of PCR cycles did not have a significant effect on BPS frequency ( $\chi^2=2.731$ ;  $p=0.435$ ).

included as a conserved region, revealed two alleles differing by a single synonymous base pair substitution. These two sequences had both been previously identified (GenBank accession numbers U34719 and U34718). Seven mutant sequences (12.1%) were identified; none were found on GenBank. Since this gene contained two sequences differing by a single BPS, we could not reliably identify chimeras. As a result, only the frequency of BPS in  $\beta_1$  and  $\beta_2$  genes were compared. The BPS frequency in the  $\beta_2$  exon (12.1%) was not significantly different from the BPS frequency in the  $\beta_1$  exon of (16.9%) at 120 PCR cycles ( $\chi^2=0.5780$ ;  $p=0.45$ ).

## DISCUSSION

In this study we analyzed MH class II  $\beta_1$  and MH class I  $\alpha_1$  sequences to provide insight into the nature of MH mutations. In both sequence libraries we discovered BPS occurring at identical sites but in different reactions. The probability of this occurring by chance is very low; therefore, it is more likely that these mutants represent somatic mutations. The criteria for acceptance of new human MHC alleles is that the sequence must be represented by two or more clones, preferably from separate reactions (Marsh et al., 2005). Applying this rule, the mutations we identified are likely somatic mutations, and their low occurrence is reflective of somatic mutations being rare events. We also found that A→G substitutions predominated over other mutations, followed by T→C mutations, in both the  $\alpha_1$  and  $\beta_1$  genes, with equal substitution rates in C and G in the  $\alpha_1$  gene. A study on somatic mutation in immunoglobulin genes of a teleost fish revealed a predominant accumulation of G·C→A·T substitutions, and this has also been shown in humans to occur as a result of the spontaneous deamination of DNA bases (Yang et al., 2006; Cooper and Youssoufian, 1988). In this study we found an accumulation of A→G

and T→C substitutions because coding DNA sequences (complementary to template) were analyzed; however, if cDNA were used we would observe the Watson-Crick complement and G→A and C→T mutations would predominate. Furthermore, there are general patterns associated with mammalian somatic mutation in immunoglobulin genes such as the approximately equal substitution rates in C and G, and a greater number of mutations in A versus T, which is what we also found in the MH genes (Smith et al., 1996; Milstein et al., 1998; Foster et al., 1999). Our analysis of the sequence library data revealed mutation clusters or “hotspots” in both the  $\beta_1$  and  $\alpha_1$  gene; although *Taq* error may be responsible for the majority of BPS, it is plausible that the MH sequence characteristics increases the likelihood of polymerase error in particular areas of the gene, and the same mechanism could be occurring *in vivo* and *in vitro*.

There are no existing studies on somatic mutations in the MH genes; however, somatic mutations in the immunoglobulin genes are well characterized (e.g. Lee et al., 2002; Rogozin et al., 2001; Shapiro et al., 2002; Yang et al., 2006). One study found that the complementarity-determining regions (CDRs) in immunoglobulins, which form antigen binding sites, have a greater frequency of somatic mutations than the framework regions (Rogozin et al., 2001). Similarly, increased somatic mutation rates in the PBR of MH would be advantageous as it would increase the MH repertoire, and could potentially be beneficial when encountering novel pathogens. However, there exists a limit to the advantages of MH allele diversity because adding new MH molecules would increase the potential for destruction of T cells that are activated by MH molecules complexed with self peptides in the thymus (Matzinger et al., 1984). There are published examples where a lack of MHC PBR polymorphism has had deleterious effects on an organism through



increasing susceptibility to pathogens (O'Brian et al., 1985; Siddle et al., 2007). Chinook salmon are anadromous and as a result they encounter different pathogen communities as they migrate from fresh water to their ocean rearing environment at about eight months of age. Allelic diversity at the MH genes may play an important role in their survival and fitness, and several studies have linked MH allelic characteristics with increased survival when encountering specific pathogens in salmonids (Arkush et al., 2002; Langefors et al., 2001). Chinook salmon have a limited number of MH loci, and we suggest that somatic mutation may be an important mechanism for increasing allelic diversity at the MH loci in Chinook salmon.

In this study, we analyzed mutation rates when amplifying the MH class II  $\beta_1$  gene in Chinook salmon under various PCR cycle numbers, and with proof-reading (Phusion<sup>®</sup>) versus non proof-reading (AmpliTa<sup>®</sup>) polymerase. We found that the polymerase used had a significant effect on the type of mutations produced, with AmpliTa<sup>®</sup> producing a greater frequency of BPS, and Phusion<sup>®</sup> producing a greater frequency of chimeras. It is not surprising that AmpliTa<sup>®</sup> produced a greater frequency of BPS since it has no proof-reading capabilities. Furthermore proof-reading polymerases have been shown to create chimera sequences at a high rate (Zylstra et al., 2008), and we found that higher numbers of PCR cycles exacerbated this problem. A study by Zylstra and colleagues (1998) showed that *Pfu*, another proof-reading polymerase, increased the formation of recombination artefacts, and they suggest reducing PCR cycle number to minimize or eliminate those artefacts (Judo et al., 1998). Additionally, Borriello and Krauter (1990) significantly reduced chimera sequence frequency using "PCR+1", which effectively eliminated/reduced PCR heteroduplexes,

and in turn sequence scrambling in *E. coli*. Although the most frequently occurring artifact mutations formed during PCR are chimeras, they are generally easily identified and eliminated. However, three chimera sequences identified in this study were found in GenBank, thereby providing evidence that undetected PCR artefacts have been mistaken for true MH PBR alleles in past studies, and more caution should be taken, particularly for highly polymorphic genes. We also found that the number of PCR cycles did not have a significant effect on the frequency of BPS. This is surprising since the proportion of mutant sequences produced is a function of the number of PCR cycles assuming that all PCR amplicons will be amplified at an equal rate. We acknowledge that PCR bias occurs, and somatic mutations likely represented a portion of mutations; nevertheless, new mutations are created in each cycle, and further amplified in subsequent cycles so we expected to observe an increase in the frequency of mutations with PCR cycle number.

The mechanism of allele diversification of the MH genes is largely unknown, and we propose that somatic mutation is a plausible mechanism for the diversification of MH alleles. This is the first study to analyze MH sequence variants in Chinook salmon for somatic mutations. In previous MH studies, sequence mutants were eliminated from the analyses unless they were common, and thus were deemed a true allele. This practice may have masked the presence of unexpected allele diversifying mechanisms such as somatic mutations. On the other hand, it is important that sequences are examined thoroughly before including them as possible alleles, since including artefacts in genetic analysis will inflate allele diversity estimates, as possibly bias estimates of other effects such as recombination (Lenz and Becker, 2008). In order to identify true alleles reliably and the most likely candidates for somatic mutation, we recommend using a proof-

reading polymerase, the lowest number of PCR cycles possible, and, ideally, conducting multiple independent PCRs for each sample. Future planned studies will determine whether the putative somatic mutations are expressed. Our findings provide insight into a possible novel mechanism acting to enhance the immune function of Chinook salmon through the creation of somatic MH diversity.

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## CHAPTER THREE

Sperm competition and MH-mediated mate choice in jack versus hooknose Chinook salmon (*Oncorhynchus tshawytscha*)



## INTRODUCTION

Sexual selection is widely recognized as a powerful evolutionary process having important consequences for reproductive behaviour, morphology, and fitness (Birkhead and Pizzari, 2002). The majority of research on sexual selection is focused on pre-copulatory mate choice; however, the discovery of female promiscuity and polyandry indicated that mate choice also persists after copulation (Birkhead and Pizzari, 2002). For males, it is well-known that promiscuity has evolved to increase reproductive fitness; however, the female benefits are less pronounced, and researchers predict that females copulate with multiple males to increase genetic diversity and fitness in offspring (Birkhead and Pizzari, 2002; Dixson and Anderson, 2001). Sexual selection occurring post-copulation is the result of two possible mechanisms: sperm competition and/or cryptic female choice (CFC). Sperm competition is competition between the sperm of multiple males to fertilize ova (Parker, 1984), and CFC occurs when the female influences the outcome of sperm competition (Eberhard, 1996). Although the mechanisms for sperm competition vary across taxa, the outcome can typically be predicted based on the knowledge of relative sperm numbers, sperm quality, the timing of inseminations, and the timing of female ovulation (Birkhead and Pizzari, 2002). In contrast, identifying CFC is challenging as it is often subtle, and masked by male-driven components (Birkhead and Pizzari, 2002).

The genes most extensively studied in mate choice research are the major histocompatibility complex (MHC) genes, presumably as a result of its crucial function in the immune system as well as its role in behavioural contexts. The MHC genes code for cell-surface receptors which bind self and non-self peptides, and present them to T cells (Lawlor et al., 1990). There are two functionally distinct MHC molecules, class I and class II, and they present peptides derived from intracellular and extracellular antigens respectively. The genes encoding the peptide binding region (PBR) of the MHC molecule are highly polymorphic and the focus of all MHC research. In mammals the MHC class I and class II genes are located in close proximity forming a linkage group or “complex” (Klein, 1986). Interestingly, in teleosts, the genes encoding the class I and class II genes are unlinked, and since they do not form a single complex they are more correctly referred to as MH genes (Stet et al., 2002). Because of the highly polymorphic nature of

the MHC PBR genes, they have been candidate genes in numerous mate choice studies. Researchers postulate that the mechanism for MHC-mediated pre-copulatory mate choice involves individual MHC-influenced body odours (in the form of soluble MHC molecules, fragments, or ligands), which females use as a cue to assess her potential partner's MHC genotype (Brown et al., 1989; Singh et al., 1987). While there have been several studies exploring the effect of MHC on pre-copulatory mate choice (reviewed in Tregenza and Wedell, 2000), there is evidence the MHC may also play a role in post-copulatory sexual selection, presumably through egg-sperm interactions (Wedekind et al., 1996; Rüllicke et al., 1998). Salmonid species are a good model system for the study of post-copulatory sexual selection, and specifically for investigating sperm competition and CFC because fertilization is external, making in-vitro fertilizations possible under controlled settings. The structure of the teleosts egg consists of an outer envelope (the chorion), and unlike mammals, only a single location for sperm entry into the egg (the micropyle; Kamler, 1992). The teleost egg offers a unique opportunity for investigating sperm competition because having only a single entrance available for fertilization should dramatically elevate sperm competition, and result in the evolution of characteristics that increase sperm competitiveness. The micropyle may also facilitate CFC. During sperm attraction and movement through the funnel-shaped micropyle, the potential exists for the egg to favor certain sperm over others, and this should lead to mate choice based on genetic compatibility at loci such as the MH genes (Skarstein et al., 2005).

Male Chinook salmon compete aggressively for spawning access to females to ensure that their sperm will fertilize the eggs (Esteve, 2005). Male Chinook salmon that mature precociously and return to freshwater at least one year earlier than other fish in the same cohort are referred to as “jacks,” while males with later maturation are referred to as “hooknose” males (Heath et al., 1994). Usually, the larger hooknose males with well-developed secondary sexual characteristics have an advantage over the smaller jacks, since the larger males guard females to prevent other males from fertilizing the eggs (Esteve, 2005). However, the smaller jacks have developed an alternative strategy to obtain fertilizations using a “sneaking” tactic (Esteve, 2005). In this tactic, jacks remain strategically hidden from the hooknose males and the females until female oviposition, when the jacks emerge from hiding, and use their small body size and cryptic colouration

to gain a position closer to the female's vent (Esteve, 2005). Research has shown that with this reproductive strategy jacks can achieve comparable paternity to that of hooknose males (Fleming and Reynolds, 2004). Although it is generally thought that jacks are the inferior male phenotype (Gross, 1984), studies have suggested that jacks have a survivorship advantage over hooknose males since exposure to ocean factors such as predators and parasites is reduced by at least one year (Gross, 1991). Because the quality of available refuges for jacks may not always be favourable for reproductive success, it is plausible that the jack phenotype would be under strong selection to evolve a strategy to enhance fertilization success through sexual selection for genetic and physical traits increasing jack sperm competitiveness.

In this study, I investigated the outcome of sperm competition between jack and hooknose male Chinook salmon in a 5x5 in-vitro fertilization experiment. Microsatellite paternity analysis revealed that jacks out-competed hooknose males, and fertilized the majority of eggs. An analysis of variance (ANOVA) conducted on these results revealed that the majority of the variance was attributed to differences in sperm traits between jack and hooknose males; however, significant interaction effects indicate a sperm-egg compatibility mechanism was also playing a role. In this study I further investigate whether genetic compatibility at the MH genes had influenced the outcome of sperm competition. I show that jacks fertilized eggs at the highest frequency when their MH class II  $\beta_1$  alleles were more similar to the female's alleles than the hooknose males. This study provides the first evidence for a substantial sperm advantage, and a novel role for the MH genotype in sperm-egg interactions during fertilization resulting from jack Chinook salmon. These results have important implications in fundamental evolutionary biology, and practical implications for the successful propagation and management of the species.

## METHODS AND MATERIALS

### **Sperm Competition**

The Chinook salmon used in this study were from the Quinsam River, British Columbia. Gametes were collected from a total of ten males and five females in reproductive condition. The males consisted of five mature males ("hooknose males") and five jacks. Jacks were distinguished from hooknose males based on their lack of

secondary sexual characteristics, gonad inspection, and body size (Heath et al. 2002). Males were dried around the vent and stripped of sperm by applying gentle abdominal pressure. Care was taken to avoid contamination with urine, mucous, and water during stripping and storage. Spermatocrit (the percentage of the milt volume occupied by the spermatozoa) was measured (Skarstein et al. 2005) for all males, and sperm density was corrected so that eggs were fertilized with the same number of sperm cells from each male. Eggs were collected by humanely euthanizing the fish and cutting the abdominal wall to release the eggs. Eggs from each female were divided into five equal groups, and sperm from a sire pair (consisting of the mixture of sperm from one jack and one hooknose male) was added to one group of eggs from each dam. This was repeated so that each sire pair fertilized one group of eggs for each dam to make a 5 x 5 factorial cross with a total of 25 maternal half-sib families. Each egg/sperm mixture was then activated by adding approximately 3X its volume of fresh water, and eggs were then incubated separately in a vertical stack incubator. The experiment was terminated when the eggs developed eyespots, and 48 eggs were selected from each cross for genetic parentage assignment and MH genotyping.

### **Parentage Assignment**

DNA was extracted from parental fin clips, as well as from embryos dissected from the eyed eggs, using a standard plate-based extraction method (Elphinstone et al., 2003). Parentage was determined for 48 offspring per cross using microsatellite markers: Ots107 (Nelson and Beacham, 1999), OtsG83b (Williamson et al., 2002), and Omy1191UW (Spies et al., 2005). Parental and offspring DNA were amplified in polymerase chain reactions (PCR) with solutions comprised of: 1.0  $\mu$ L of extracted DNA, 0.50  $\mu$ L of each primer (100 ng/ $\mu$ L), 2.5  $\mu$ L 10x reaction buffer, 3.0  $\mu$ L MgCl<sub>2</sub> (25mM), 1.0  $\mu$ L dNTP's (200  $\mu$ M), 0.20 U AmpliTaq<sup>®</sup> polymerase (Applied Biosystems, Foster City, CA), and ddH<sub>2</sub>O to make a 25  $\mu$ L reaction. The reaction profile consisted of: 2 min. initial denaturation (95°C), 30 cycles of 30 sec. denaturation (95°C), 30 sec. annealing (52-60 °C), 1 min. extension (72°C), followed by a final 10 min. extension (72°C). The forward primers for all PCRs were dye-labeled, and products were analyzed on a LI-COR 4300 DNA analyzer. Gene ImagIR software was used to visually score and determine

fragment sizes which were then assigned to the dam and either the jack or hooknose males.

### **MH genotyping**

The PBR of the MH class II  $\beta_1$  gene was PCR amplified from parental samples with primers developed by Docker and Heath (2002). The sequence of the sense primer was 5'-CCG ATA CTC CTC AAA GGA CCT GCA-3', and the anti-sense primer was 5'-GGT CTT GAC TTG MTC AGT CA-3', and amplified a 294 bp fragment.. The PCR consisted of: 1.0  $\mu$ L extracted DNA (50-100 ng), 0.50  $\mu$ L of each primer (100 ng/ $\mu$ L), 2.5  $\mu$ L 10x reaction buffer, 3.0  $\mu$ L MgCl<sub>2</sub> (25mM), 1.0  $\mu$ L dNTP's (200  $\mu$ M), and 0.20 U AmpliTaq<sup>®</sup> polymerase (Applied Biosystems), and ddH<sub>2</sub>O to make a 25  $\mu$ L reaction. The reaction profile consisted of: 2 min. initial denaturation (95°C), 30 cycles of 30 sec. denaturation (95°C), 30 sec. annealing (52°C), 1 min. extension (72°C), followed by a final 10 min. extension (72°C). The MH class II  $\beta_1$  gene was not genotyped in the offspring due to limited parental allele variation (see Results section, below).

The MH class I  $\alpha_1$  gene was PCR amplified from parental and offspring samples using primers previously developed (Miller et al., 1997). The sequence of the sense primer was 5'-TGA CTC ACG CCC TGA AGT A-3', and the anti-sense primer was 5'-CTC CAC TTT GGT TAA AAC G-3'. The reaction and cycling conditions were identical to those used for amplification of the  $\beta_1$  gene (see above).

MH PCR products (both MH class I and II) from parental samples were cloned into a pGEM<sup>®</sup>-T vector following the manufacturer's protocol (Promega). White colonies were selected, boiled in ddH<sub>2</sub>O, and then used for insert verification PCR. The insert was amplified using the M13 forward primer (5'-GTA AAA CGA CGG CCA GT-3') and M13 reverse primer (5'-AAA CAG CTA TGA CCA TG-3') under the following conditions: 2 min. initial denaturation (94°); 35 cycles of 1 min. denaturation (94°), 1 min. annealing (55°), 1 min. extension (72°); and a final 3 min. extension cycle (72°). Each 25  $\mu$ L reaction consisted of: 50-100 ng plasmid DNA, 0.50  $\mu$ L of each primer (100 ng/ $\mu$ L), 2.5  $\mu$ L 10x reaction buffer, 25 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTPs, and 0.50 U AmpliTaq<sup>®</sup> polymerase (Applied Biosystems). Eight sub-clones containing appropriately- sized inserts were sequenced from each parent. PCR products were purified using AMPure (Agencourt) purification system, and sequencing reactions were

performed using the M13 forward primer along with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). After purification using CLEANseq (Agencourt), sequencing was performed using the M13 forward primer and ABI's BigDye Terminator version 3.1 on an ABI 3130xl sequencer.

To evaluate the inheritance of the MH alleles in the F1 generation, a subset of the offspring from each of the 25 maternal half-sib families were genotyped at the MH class I  $\alpha_1$  gene. Offspring MH class I  $\alpha_1$  PCR products were directly sequenced, and allelic variation was read from the chromatogram. The MH class II  $\beta_1$  gene lacked sufficient polymorphism amongst the parents to allow meaningful segregation analysis, and, therefore, offspring were not genotyped at the MH class II  $\beta_1$  locus.

## DATA ANALYSIS

### **Analysis of reproductive success**

The frequency of jack-sired and hooknose-sired offspring was determined for each family, and cross tabulation (SYSTAT, version 12) was used to determine whether the number of jack sired offspring differed significantly from the number of hooknose sired offspring. An ANOVA was used to partition the variance in male mating success into dam, sire-pair, and dam-sire interaction effects.

### **Analysis of MH sequences and segregation**

**Parental genotypes:** Geneious Pro software (Biomatters) was used to create alignments to identify alleles. Two alignments, one each for the  $\alpha_1$  and  $\beta_1$  gene, were built using all sequences from parental samples. Actual alleles occurred at a greater frequency than PCR artifacts, and sequences were verified by comparison to alleles identified in the previous project. Identical sequences were grouped and assigned an allele name.

**Offspring genotypes:** Each maternal half-sib family consisted of a dam and two possible sires (either the jack or hooknose male); fifteen to 34 offspring were genotyped from each maternal half-sib family for the  $\alpha_1$  gene. Sequence scanner version 1.0 (Applied Biosystems) was used to view overlapping base peaks in sequences obtained from offspring PCR products for the  $\alpha_1$  gene. The overlapping peaks identified in offspring sequences were examined, and compared to the nucleotide polymorphisms identified in parental sequences to deduce offspring genotype.

**Segregation analysis:** Parental alleles are expected to occur at 50% frequency in the offspring, based on Mendel's principle of segregation. To determine whether offspring were inheriting maternal and paternal  $\alpha_1$  alleles as expected by Mendel's principle, a chi-square test for each family was conducted to detect significant departures from the 1:1 ratio. Because of low sample size, a chi-square analysis could not be used to establish whether offspring genotype frequencies departed significantly from the expected Mendelian ratios.

#### **Analysis of offspring genotypes**

The purpose of the following analyses is to determine whether the egg-sperm compatibility is affected by whether the sperm MH genotypes are more similar or dissimilar to the egg.

Expected offspring  $\alpha_1$  and  $\beta_1$  genotypes were determined for all parental crosses based on Mendelian segregation. An allele amino acid dissimilarity index (AADI) was estimated for expected offspring genotypes as the number of amino acid differences between the two allele sequences. Thus, a higher number represents greater dissimilarity between alleles, and homozygotes would have a dissimilarity of zero. For each maternal half-sib family, the four AADI values were used to determine an average value. For each maternal half-sib family the AADI average obtained from jack-sired offspring genotypes was subtracted from the AADI average from hook-nose sired offspring, and this value was used to determine whether AADI was associated with higher/lower jack fertilization success. A scatter-plot was created by plotting the frequency of jack-sired offspring against the difference in the average AADI for each family; regression was used to determine whether the former was dependant on the latter. This analysis was conducted for both the  $\alpha_1$  and  $\beta_1$  gene.

## **RESULTS**

### **Reproductive success**

Parentage analysis revealed that jacks had sired the majority of offspring in all half-sib families, and in male pair 5, jacks had sired all offspring (Figure 1). The

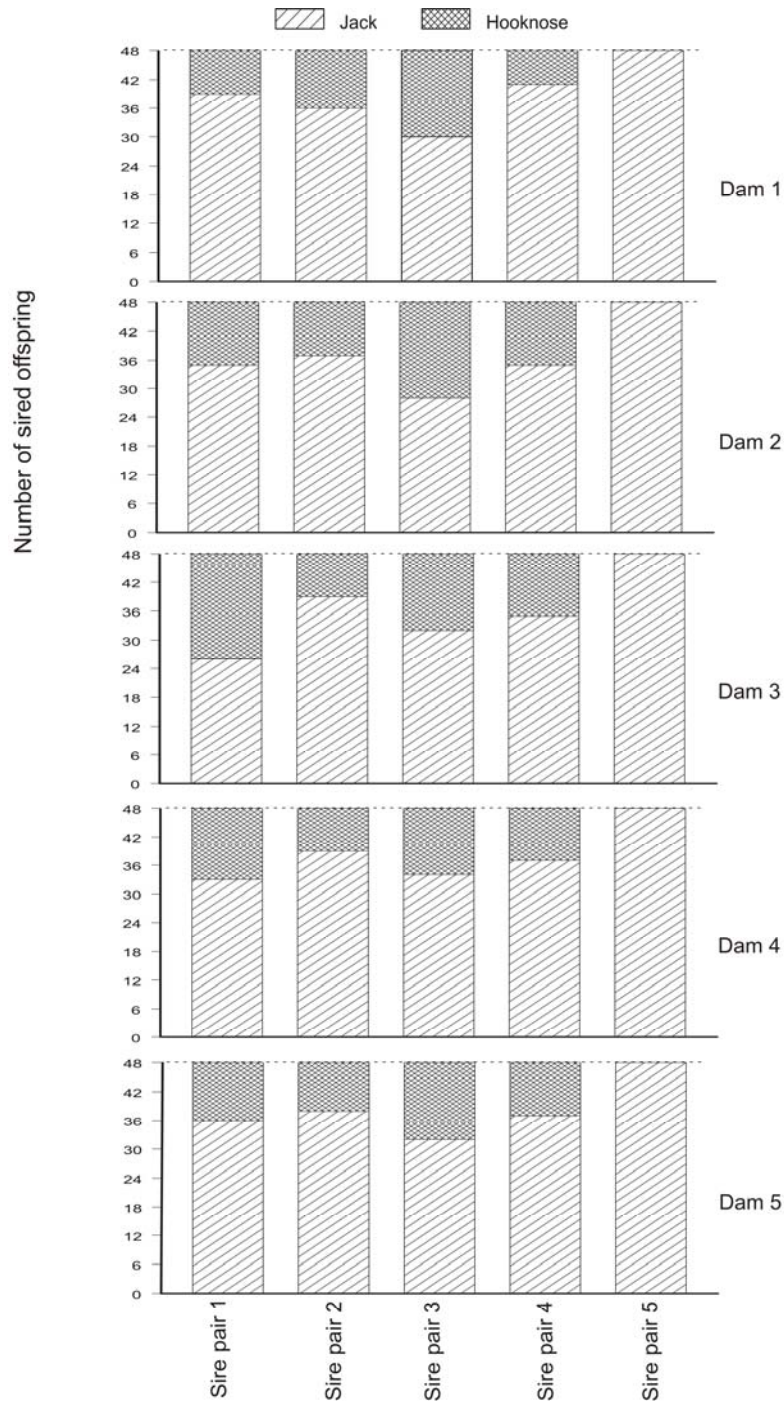


Figure 1. Microsatellite analysis was used to determine parentage of 48 Chinook salmon offspring per maternal half-sib family. Each bar displays the number of jack-sired and hooknose-sired offspring in each half-sib family. The difference between the number of jack-sired offspring, and the number of hooknose-sired offspring across all families was highly significant ( $\chi^2=132.6$ ;  $p<0.0010$ ).



difference between the number of jack-sired offspring, and the number of hooknose-sired offspring was highly significant ( $\chi^2=133$ ;  $p<0.0010$ ). An ANOVA revealed a significant dam effect ( $F=3.18$ ;  $p=0.013$ ), a significant sire-pair effect ( $F=23.2$ ;  $p<0.0010$ ), and a significant dam-sire pair interaction ( $F=2.53$ ;  $p=0.0010$ ).

### **MH sequence and segregation**

A total of four MH II  $\beta_1$  alleles were identified in parental samples, B1-Ots-A, B1-Ots-B, B1-Ots-C, and B1-Ots-D, and all nucleotide sequences are found in GenBank (accession numbers: AY100006, EF432120, DQ450874, and AY100008 respectively; Table 1). Two sire pairs had identical genotypes, and four of five females were homozygous for the U80299 allele. Due to the lack of allelic diversity at this gene in the parental samples, offspring were genotyped for the MH I  $\alpha_1$  gene only, which was found to be more polymorphic, thus making it possible to determine inheritance of maternal and paternal alleles.

A total of 13 MH I  $\alpha_1$  alleles were identified in parental samples, and of these only four were found in the GenBank database (Table 1). A1-Ots-N, A1-Ots-T, A1-Ots-H, and A1-Ots-L are found in GenBank under the accession numbers DQ647922, U80293, DQ647923, and U80284 respectively. Translation of the 13 nucleotide sequences revealed that all base substitutions are non-synonymous, and thus form distinct amino acid sequences (Table 1). A1-Ots-N, A1-Ots-E, and A1-Ots-T are alleles found only in jacks, and A1-Ots-D, A1-Ots-C, and A1-Ots-I were found only in hooknose males (Table 1). The alleles occurring at the greatest frequency were A1-Ots-P (17.6%), followed by A1-Ots-R (15.7%), A1-Ots-A (12.6%), and A1-Ots-M (12.4%; see Table 1).

Analysis of the segregation of paternal and maternal  $\alpha_1$  alleles in jack-sired offspring revealed no departure from the 1:1 ratio (too few offspring were sired by hooknose males to allow an assessment of segregation in the hooknose offspring).

### **Offspring Genotypes**

To determine whether jack sperm were more successful at fertilization when more genetically similar/dissimilar to the ovum at the MH gene than the hooknose male, a regression analysis was performed. The frequency of jack-sired offspring was not dependant upon the difference in average AADI between jack and hooknose offspring expected genotypes for the  $\alpha_1$  gene ( $F = 0.0129$ ;  $p = 0.911$ ; Figure 2a). For the  $\beta_1$  gene,

Table 1. Alleles identified in the Chinook salmon study population for the MH class I  $\alpha_1$  and MH class II  $\beta_1$  gene. J denotes the jack male, H the hooknose male, and D the dam, and indicates in which parental sample the alleles were identified. The frequency was determined by counting the occurrence of that allele in the entire population, and dividing by 2N (N=483). Since offspring were not genotyped for the  $\beta_1$  gene, a frequency is not available.

Allele	Fish	Frequency	Amino Acid Sequence	GenBank No.
A1-Ots_A	D1,D3,H4	0.126	FYTASSEVPNFPEFVTVGMVDGVQMVHYDSNSQRAVPKQDWINKAAETLPQYWESETGNFKGAQQTTFKANIDIVKQ	NA
A1-Ots-R	D2,D5,H4	0.157	FYTASSEVPNFPEFVVGMVDGVQMVHYDSNSQRAVPKQDWINKAAD--PQYWERNTGNFKGDQQTTFKANIDIAKQ	NA
A1-Ots-I	H1	0.034	FYTASSEVPNFPEFVVGMVDGVQMVHYDSNSQRAVPKQDWINKAAD--PQYWERNTGTFKGNQQTTFKANIDIAKQ	NA
A1-Ots-C	H2	0.023	FYTASSEVPNFPEFVVGMVDGVQMVHYDSNSQRAVPKQDWINKAAETLPQYWERETGTFKGDQQTTFKANIDIAKQ	NA
A1-Ots-E	J2	0.034	FYTASSEVPNFPEFVVGMVDGVQMVHYDSNSQRAVPKQDWINKAAETLPQYWERNTGNFKGAQQTTFKANIDIAKQ	NA
A1-Ots-N	J3,J4	0.062	FYTASSEVPNFPEFVVGTVDGVQMFHYDSNSQRAVPKQDWINKAAETLPQYWERETGICKGTQQTTFKANIDIVKQ	DQ647922
A1-Ots-T	J5	0.034	FYTASSEVPNFPEFVVGTVDGVQMFHYDSNSQRAVPKQDWINKAAD--PQYWERNTGNCKGTQQTTFKANIDIVKQ	U80293
A1-Ots-L	D5,H5	0.046	FYTASSEVPNFPEFVVGTVDGVQMFHYDSNSQRAVPKQDWINKAAETLPQYWERETGIDKGAQQTTFKANIDIVKQ	U80284
A1-Ots-P	D4,J3,J5,H2, H3,H5	0.176	FYTASSEVPNFPEFVVGMVDGVQMFHYDSNSQRAVPKQDWINKAAETLPQYWERETGNCKGDQQTTFKANIDIAKQ	NA
A1-Ots-D	H1	0.032	FYTASSEVPNFPEFVVGMVDGVQMVHYDSNSQRAVPKQDWINKAAD--PQYWERNTGNCKGDQQTTFKANIDIVKQ	NA
A1-Ots-H	D3	0.057	FYTASSEVPNFPEFVVGMVDGVQMFHYDSNSQRAVPKQDWINKAAETLPQYWERNTGNCKGDQQTTFKANIDIVKQ	DQ647923
A1-Ots-M	D4,J1,J4	0.124	FYTASSEVPNFPEFVTVGMVDGVQMVHYDSNSQRAVPKQDWINKAAD--PQYWERNTGNFKGAQQTTFKANIDIAKQ	NA
A1-Ots-B	D1,J2	0.085	FYTASSEVPNFPEFVVGMVDGVQMVHYDSNSQRAVPKQDWINKAAETLPQYWESETGNFKGAQQTTFKANIDIVKQ	NA
B1-Ots-A	D1-5, H1-5, J1-5	NA	GIEFIDSYVFNKAETIRFNSTVGRYVGYTELGVKNAEAWNKGQQLGQEQAELERFCKPNAALHYRAILDK	AY100006
B1-Ots-B	D4	NA	GIEFIDSYVFNKVEHIRFNSTVGRYVGYTELGLKNAEAWNKGQQLGQEQAELERFCKPNAALHYRAILDK	EF432120
B1-Ots-C	H2	NA	GIEFIDSYVFNKVENIRFNSTVGRYVGYTELGVKNAEAWNKGQQLGQEQAELERFCKPNAALHYRAILDK	DQ450874
B1-Ots-D	H5, J4	NA	GIEFIHSYVFNKVEHIRFNSTVGRYVGYTELGLKNAEAWNKGQQLGQEQAELERVCKPNAALHYRAILDK	AY100008

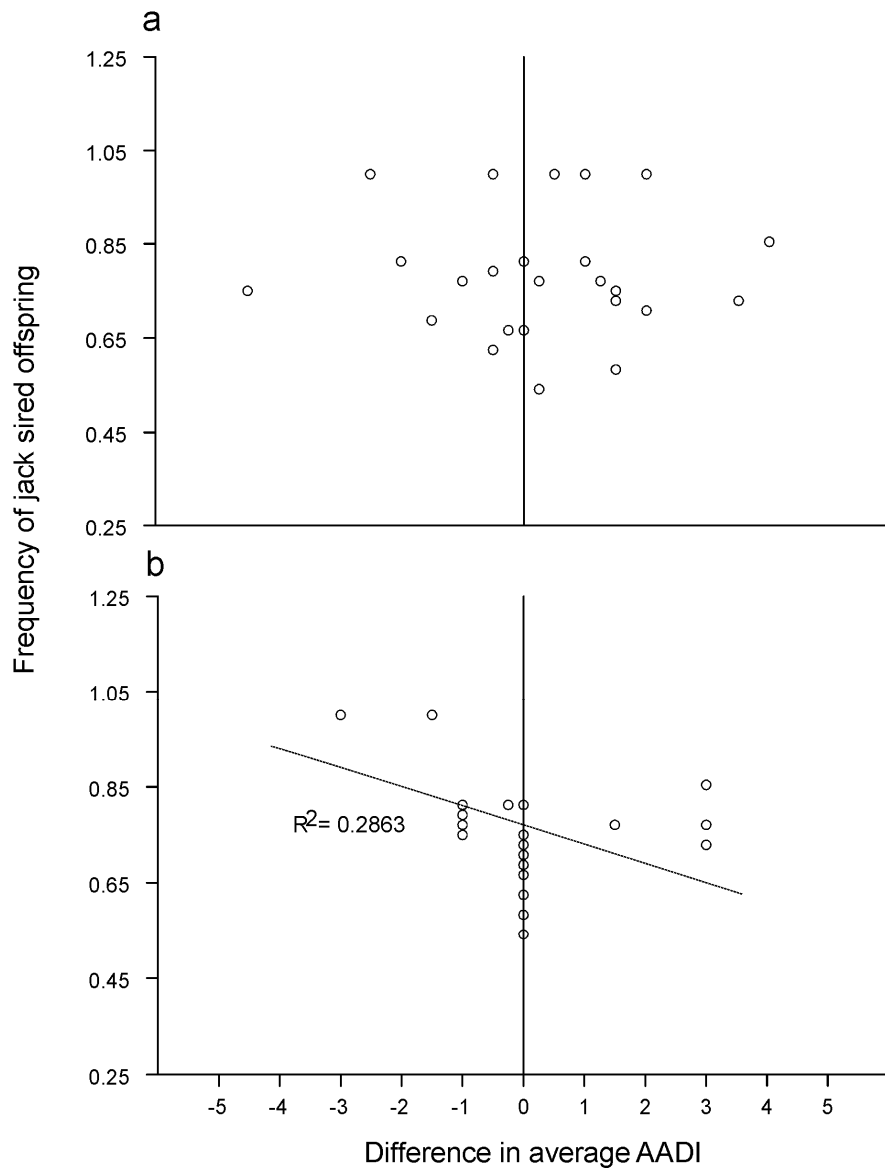


Figure 2. The frequency of jack sired offspring versus the difference in the average amino acid dissimilarity index (AADI) in jack and hooknose offspring per maternal half-sib family. The average AADI was determined from the individual AADI values obtained from expected genotypes in offspring in each maternal half-sib cross. The frequency of jack-sired offspring was not dependant upon the difference in average AADI between jack and hooknose offspring for a) the  $\alpha_1$  gene ( $F = 0.0129$ ;  $p = 0.911$ ); however, this relationship was significant for b) the  $\beta_1$  gene ( $F = 9.23$ ;  $p = 0.006$ ).

when jack sperm were more genetically similar to the female ovum than the hooknose sperm, they sired offspring at a higher frequency, and this relationship was significant ( $F = 9.23$ ;  $r^2 = 0.2863$ ;  $p = 0.006$ ; Figure 2b).

## DISCUSSION

In this study jack sperm out-competed hooknose sperm, and fertilized the majority of eggs in 25 maternal half-sib families. The elevated jack fertilization success is not surprising since studies on other salmonid species have shown that sperm from precociously maturing males is higher in quality than the sperm from the dominate male phenotype. For instance, Gage and colleagues (1995) investigated sperm characteristics in alternative male reproductive strategies in Atlantic salmon (*Salmo salar*), and discovered that precociously maturing parr produced more motile and long-lived sperm than anadromous males. Similar findings were observed in rainbow trout (*Salmo gairdneri*), sticklebacks (*Gasterosteidae*), and in other Atlantic salmon studies (Linhart, 1984; de Fralpont, 1993; Vladic and Jarvi, 2001; Stoltz and Neff 2006). My ANOVA revealed a highly significant male pair effect which likely reflects differences in sperm swimming velocity between jack and hooknose males. Jack fertilization success did vary by female; nevertheless, jack sperm sired the majority of offspring in all families indicating that on average jack sperm was more effective than hooknose sperm, likely due to sperm velocity. In Chinook salmon, preliminary data has shown that jack sperm swimming velocity did not differ significantly from hooknose sperm swimming velocity five seconds after activation (T. Pitcher, University of Windsor, unpublished data). This indicates that there may be unexplored factors associated with increased sperm quality in jacks apart from swimming speed.

I found that the dam component in the ANOVA had a significant effect on the relative success of the jack sire in the jack/hooknose pair. The dam component of the variance in jack fertilization success reflects differences between the eggs of each female that affect jack fertilization success, such as the size of the eggs, the amount of ovarian fluid surrounding the eggs, or even the chemical composition of the egg and/or ovarian fluid. Female cryptic choice is female choice exerted post-copulation (Eberhard 1996). In salmonids, researchers have discovered that ovarian fluid released with the female's eggs

can influence sperm behaviour, and is a putative mechanism for CFC (Lahnsteiner 2002; Gage et al. 2004; Rosengrave et al. 2009). Rosengrave et al. (2008) analyzed CFC in Chinook salmon, and found that the composition of female ovarian fluid differentially enhanced sperm swimming speed among males. Although ovarian fluid was not controlled for between females in this study, the fertilization medium consisted of mostly water, and ovarian fluid would have been too dilute to contribute significantly to sperm swimming speed. Furthermore, the female variance only represented 0.96% of the total variance; thus, although interesting, ovarian fluid as a mechanism for CFC is likely not a major component of the observed variance in fertilization success in this study.

In this study I found a significant dam x sire-pair interaction that reflects egg-sperm compatibility variation among families. Analysis of the class I  $\alpha_1$  gene revealed high allelic polymorphism; however, mate choice was not occurring for this gene. However, for the  $\beta_1$  gene, greater fertilization success occurred when jack sperm carried alleles that were more similar to the female's allele than the hooknose sperm. Contrary to studies that demonstrate mate choice promoting genetic diversity, in this study mate choice resulted in a decline in polymorphism at the  $\beta_1$ , which is already considerably less polymorphic than the  $\alpha_1$  gene in Chinook salmon (Miller and Withler 1998). The class I and class II MH genes are unlinked in salmon, and thus can evolve independently, perhaps explaining the difference in polymorphism at the two genes. Mate choice for higher genetic similarity is a mechanism for hybridization avoidance or for limiting outbreeding depression. Limiting hybridization may be adaptive as several studies have shown that interbreeding between hatchery and wild fish significantly reduces productivity and viability of the naturally spawning population (reviewed in Reisenbichler and Rubin 1999). Alternatively, local adaptation may drive selection for specific alleles rather than heterozygosity to enhance immunity when encountering common pathogens. My finding is similar to the results of another study on Atlantic salmon (*Salmo salar*), which also found that males won more fertilizations when they more genetically similar at the MH gene to females; however, this was discovered for the MH class I locus (Yeates et al. 2009). Alternatively, another non-MH based mechanism may be driving the significant interaction such as a receptor-ligand binding interaction at the egg and sperm cell surface. In the sea urchin, genus *Echinometra*, the protein bindin

mediates sperm attachment to eggs, and, similar to the MH molecules, bindin is highly polymorphic (Palumbi 1999). One study demonstrated that sea urchin eggs discriminated amongst different bindin molecules, and chose sperm from the male with which they shared the largest number of bindin alleles (Palumbi 1999). Teleosts lack the protein bindin; however, it is plausible that other unidentified polymorphic molecules play a similar function in promoting assortative mating in salmon.

The outcome of sperm competition in salmonids is largely dependant on sperm traits such as sperm velocity and longevity (Gage et al. 2004), and here I provide evidence for a bias towards fertilization success in a specific male life history in *in vitro* sperm competition. Such factors are critically important in artificial fertilizations where natural pre-zygotic male-male competition and behavioural female choice are eliminated. Wedekind et al. (2007) argued that mixed-milt fertilizations can result in artificial selection for life history traits in salmonids due to variance in reproductive success between male life histories. In this study I showed that jacks had significantly greater reproductive success than hooknose males. Since there is evidence for a strong additive genetic component to jacking rates (Heath et al. 1994), one would expect that selection for jacks would occur over a relatively short period of time under hatchery conditions. Furthermore, the increased variance in male reproductive success in artificial fertilizations can increase probability of inbreeding and increase loss of genetic variation (Wedekind et al. 2007). Although CFC may operate to negate the above mentioned genetic consequences, my study showed that male sperm quality was the main determinate of post-copulatory reproductive success. In order to reduce genetic change in future populations, supportive breeding programs need to implement fertilization protocols that mimic reproduction occurring in the wild; however, this would be near impossible and the population genetics of eggs fertilized artificially will always differ from the one of eggs fertilized naturally in the wild (Campton 2005; Quinn 2005; Wedekind et al. 2007). Nevertheless, hatcheries play a crucial role in management and conservation of salmon, as human demand far exceeds the number of fish natural habitats alone can supply; therefore, it is of great importance that more genetically benign management strategies are implemented (Campton 2005).

In this study jacks had higher reproductive output than hooknose males in all breeding experiments, and this result is attributed to differences in sperm quality, such as sperm swimming velocity and longevity, between jack and hooknose males. I also found subtle differences in ovarian fluid or egg composition between the females; however, the significant interaction effect can only be explained by CFC mediated by the egg itself. Analysis for genetic compatibility revealed that CFC for MH  $\alpha_1$  gene had not occurred; however, when jacks were more similar at the  $\beta_1$  gene to the female in comparison to the hooknose male, jack fertilization success increased. These results demonstrate that mixed-milt fertilizations lead to artificial selection for the jack life history, and mate choice occurring at the sperm-egg level can also contribute to male reproductive success. This is the first study to have analyzed post-copulatory sexual selection in jack and hooknose Chinook salmon, and my results provide novel insight into the evolutionary mechanisms of sperm competition and CFC occurring in the alternate male phenotypes. These mechanisms have important evolutionary as well as management implications, which with further research are valuable tools for predicting population dynamics and implementing management strategies.

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## CONCLUSIONS AND FUTURE DIRECTIONS

A library consisting of MH class I  $\alpha_1$  and class II  $\beta_1$  sequences for Chinook salmon (*Oncorhynchus tshawytscha*) was surveyed in Chapter two, and analysis of the base pair substitutions revealed mutation clusters or “hotspots” on the MH gene. Also, identical mutations occurring in independent PCR reactions and fish were observed. This suggests that point mutation is occurring in somatic cells, and is the mechanism for the diversification of MH alleles in Chinook salmon. A series of PCR-based experiments were conducted to investigate the effects of PCR conditions on the observed mutation frequency. I found that chimera mutations were produced at the highest frequency, and were elevated with increased PCR cycles and when using proof-reading enzyme. Base pair substitutions also increased in frequency when using a non-proofreading polymerase; however, the number of PCR cycles did not affect the frequency of point mutations with this enzyme. To reduce the frequency of PCR artefacts I suggest that proof-reading enzyme is used along with the lowest number of PCR cycles necessary. The results of Chapter two provided the basis for proper MH allele identification, and were applied in Chapter three.

In order to provide more evidence for somatic mutation as a mechanism with which MH alleles diversify in Chinook salmon, it would be beneficial to detect the cDNA copies of the sequence variants identified in genomic DNA. This has already been attempted in the Heath lab; however, cDNA copies of the gDNA variants were not detected. Somatic mutation produces a genetic mosaic, meaning the mutation occurs in some cells but not others, and, as a result, detection of the somatic mutation is challenging especially when it is a rare event. I suggest using PCR product obtained with guidelines outlined in Chapter two to reduce PCR artefacts in a more accurate and high-throughput sequencing method for detection of mutants, such as pyrosequencing. Somatic mutation occurs in antibodies when B cells are stimulated by antigen and helper T cells, and this accumulation of point mutations is known as affinity maturation. It would be interesting to analyze MH sequences in fish subjected to a disease challenge to investigate whether antigens also stimulate point mutations in the MH genes.

The outcome of sperm competition between jack and hooknose males was analyzed in 25 maternal half-sib families, and I found that the number of jack-sired

offspring significantly exceeded the number of hooknose-sired offspring in all families. Analysis of the variance in male success revealed significant dam effects, sire pair effects, and sire pair-dam interaction effects. The sire variance represented the greatest portion of the total variance, and is most likely attributed to differences in jack sperm swimming velocity versus hooknose sperm swimming velocity. A significant sire pair-dam interaction effect indicated that cryptic female choice had occurred. Further investigation revealed that jacks sired a higher frequency of offspring when their MH class II  $\beta_1$  alleles were more similar to the female's allele. This is contrary to the expectation of mate choice occurring for genetically dissimilar males; however, these results suggest that mate choice is occurring for particular alleles that provide an adaptive advantage to jacks.

In order to gain further insight on post-copulatory mate choice in Chinook salmon, it would be useful to repeat the experiment using wild fish. This would allow us to see whether mate choice is also occurring for more similar MH  $\beta_1$  alleles in wild jacks. If so, it would strongly suggest that mate choice for more similar  $\beta_1$  alleles is not only adaptive for hatchery fish, but also gives an adaptive advantage for jacks in their natural habitat. Also, it would be of interest to determine whether MH-related proteins or other polymorphic proteins are being expressed in the egg and sperm, and whether they function in mate choice. From a conservation standpoint, it is crucial that hatchery managers develop fertilization methods to reduce sperm competition as my results have indicated that a) there is high variance in reproductive success of males, and b) sperm competition can lead to a decrease in genetic diversity at crucial genes such as the MH genes.

The results of this work have important fundamental and applied implications to science. From an evolutionary standpoint, these results provide preliminary information on a key evolutionary process functioning at the MH genes. Somatic mutation at the MH genes would revolutionize our thinking on how the MH genes evolve in not only salmon, but in other species. From a conservation standpoint, improvement of existing fertilization protocols is necessary to maintain genetic diversity at the MH gene, and ensure the survival of fish in the wild. These results provide valid reason as to why more genetic insight is needed in hatcheries.

## APPENDIX A

Mutations identified in the Chinook salmon MH sequence libraries for the MH I  $\alpha_1$  and MH II  $\beta_1$  genes (Chapter two), their location on the gene (read complementary to sense primer i.e. in the 3' to 5' direction), the frequency observed, and the nucleotides flanking the base substituted.

MH Gene	Mutation Position	Base Substitution	Number of	
			Independent PCR reactions	Flanking Sequence
class I $\alpha_1$	84	T→C,A	3	TATGA
	128	A→G,C	3	AAACA
	100	A→G	2	AGAGA
	193	C→T	2	AGCAG
	194	A→G,T	2	GCAGA
	3	C→T	1	TTCTA
	9	C→T	1	ACCGC
	15	T→A	1	TCTTC
	18	T→C	1	TCTGA
	19	G→T	1	CTGAA
	31	T→C	1	ACTTC
	39	G→A	1	GAGTT
	41	A→G	1	GTTCG
	42	C→G	1	TTCGT
	46	G→T	1	TGGTT
	48	T→C	1	GTTGT
	50	T→C	1	TGTGG
	53	G→A	1	GGAGA
	55	A→G	1	GGATG
	58	G→A	1	TGGTG
	68	T→C	1	TGTTC
	69	T→C	1	GTTCA
	73	A→G	1	AGATG
	76	T→C	1	TGTTT
	77	T→C	1	GTTTC
	77	T→C	1	GTTTC
	88	A→G	1	ACAGC
	91	A→T	1	GCAAC
	94	A→G	1	ACAGC
	98	A→G	1	CCAGA
	112	A→C	1	CCAAA
	113	A→G	1	CAAAC
	115	C→T	1	AACAG
	119	A→G	1	GGACT
	123	G→A	1	TGGGT
	129	C→T	1	AACAA
	131	A→G	1	CAAGG
	133	G→A	1	AGGCA
	142	A→C	1	AAACA
	143	C→A	1	AACAC
	148	C→T	1	TGCCA

	152	A→G	1	AC <u>A</u> GT
	152	A→G	1	AC <u>A</u> GT
	166	A→T	1	GG <u>A</u> AC
	200	T→C	1	TT <u>T</u> CA
	209	A→T	1	CA <u>A</u> CA
	214	G→T	1	TC <u>G</u> AT
	215	A→G	1	CG <u>A</u> TA
	217	A→G	1	AT <u>A</u> TT
	219	T→C	1	ATT <u>G</u> T
	223	A→C	1	TAA <u>A</u> G
class II $\beta_1$	211	A→G	3	AG <u>A</u> CA
	19	T→C	2	AC <u>T</u> CT
	31	A→G	2	TCA <u>A</u> T
	35	A→T,G	2	TA <u>A</u> GG
	36	G→T,A	2	AA <u>G</u> GC
	61	A→G	2	GC <u>A</u> CT
	78	T→C	2	GT <u>T</u> GG
	114	A→G	2	GC <u>A</u> TG
	128	C→T	2	TC <u>C</u> TC
	149	C→T	2	GG <u>C</u> GG
	10	T→C	1	AG <u>T</u> TT
	12	T→C	1	TT <u>T</u> AT
	16	C→T	1	TAC <u>A</u> C
	17	A→G	1	AG <u>A</u> CT
	22	T→C	1	CTT <u>A</u> T
	23	A→G	1	TT <u>A</u> TG
	24	T→C	1	TAT <u>G</u> T
	27	T→C	1	GT <u>T</u> TT
	32	A→G	1	CA <u>A</u> TA
	42	A→G	1	GA <u>A</u> TA
	44	A→G	1	ATATA
	49	A→G	1	TCAGA
	50	G→T	1	CAGAT
	53	T→C	1	ATTCA
	57	C→T	1	AACAG
	77	T→C	1	TGTTG
	80	G→T	1	TGGAT
	81	A→G	1	GGATA
	89	A→T	1	TGAGC
	97	T→C	1	GTGTG
	98	T→C	1	TGTGA
	100	A→G	1	TGAAG
	101	A→G	1	GAAGA
	104	A→G	1	GAATG
	108	A→G	1	GCAGA
	109	G→A	1	CAGAA
	119	A→G	1	GAACA
	121	A→G	1	ACAAA
	122	A→G	1	CAAAG
	123	A→C	1	GCACT
	127	C→T	1	GTCCT
	134	T→C	1	GCTGG
	139	T→C	1	GTCAA

141	A→G	1	CAAGA
146	A→C	1	GCAGG
152	A→G	1	GGAGC
163	T→C	1	GTTTC
164	T→C	1	TTTCT
169	T→A	1	GTAAG
177	C→T	1	AACGC
184	C→T	1	CTCTC
185	C→T	1	TCTCC
195	A→G	1	AGAGC
196	G→A	1	GAGCC
197	C→T	1	AGCCA
208	A→C	1	ACAAG
211	A→G	1	AGACA
213	A→G	1	ACA

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## APPENDIX B

The nucleotide sequences of the 13 MH class I  $\alpha_1$  alleles found in the study population of Chinook salmon from Chapter three. Nucleotide differences are in grey.

	1	10	20	30	40	50	60
Consensus	TTCTACACCGCATCTTCTGAAGTTCCCAACTTCCCAGAGTTCGTGGTTGTGGGGATGGTG						
Al-Ots-M	TTCTTACACCGCATCTTCTGAAGTTCCCAACTTCCCAGAGTTCGTGATTGTGGGGATGGTG						
Al-Ots-H	TTCTTACACCGCATCTTCTGAAGTTCCCAACTTCCCAGAGTTCGTGGTTGTGGGGATGGTG						
Al-Ots-D	TTCTTACACCGCATCTTCTGAAGTTCCCAACTTCCCAGAGTTCGTGGTTGTGGGGATGGTG						
Al-Ots-P	TTCTACACCAACATCTTCTGAAGTTCCCAACTTCCCAGAGTTCGTGGTTGTGGGGATGGTG						
Al-Ots-L	TTCTACACCGCATCTTCTGAAGTTCCCAACTTCCCAGAGTTCGTGGTTGTGGGGACGGTG						
Al-Ots-T	TTCTACACCGCATCTTCTGAAGTTCCCAACTTCCCAGAGTTCGTGGTTGTGGGGACGGTG						
Al-Ots-N	TTCTTACACCGCATCTTCTGAAGTTCCCAACTTCCCAGAGTTCGTGGTTGTGGGGACGGTG						
Al-Ots-E	TTCTACACCGCATCTTCTGAAGTTCCCAACTTCCCAGAGTTCGTGGTTGTGGGGATGGTG						
Al-Ots-C	TTCTACACCGCATCTTCTGAAGTTCCCAACTTCCCAGAGTTCGTGGTTGTGGGGATGGTG						
Al-Ots-I	TTCTACACCGCATCTTCTGAAGTCCCAACTTCCCAGAGTTCGTGGTTGTGGGGATGGTG						
Al-Ots-R	TTCTACACCGCATCTTCTGAAGTCCCAACTTCCCAGAGTTCGTGGTTGTGGGGATGGTG						
Al-Ots-A	TTCTACACCGCATCTTCTGAAGTTCCCAACTTCCCAGAGTTCGTGATTGTGGGGATGGTG						
Al-Ots-B	TTCTACACCGCATCTTCTGAAGTTCCCAACTTCCCAGAGTTCGTGGTTGTGGGGATGGTG						
Consensus	GATGGTGTTTCTAGATGGTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACAGGAC						
Al-Ots-M	GATGGTGTTTCTAGATGGTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACAGGAC						
Al-Ots-H	GATGGTGTTTCTAGATGTTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACAGGAC						
Al-Ots-D	GATGGTGTTTCTAGATGGTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACAGGAC						
Al-Ots-P	GATGGTGTTTCTAGATGTTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACAGGAC						
Al-Ots-L	GATGGTGTTTCTAGATGTTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACAGGAC						
Al-Ots-T	GATGGTGTTTCTAGATGTTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACAGGAC						
Al-Ots-N	GATGGTGTTTCTAGATGTTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACAGGAC						
Al-Ots-E	GATGGTGTTTCTAGATGGTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACAGGAC						
Al-Ots-C	GATGGTGTTTCTAGATGGTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACAGGAC						
Al-Ots-I	GATGGTGTTTCTAGATGGTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACAGGAC						
Al-Ots-R	GATGGTGTTTCTAGATGGTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACAGGAC						
Al-Ots-A	GATGGTGTTTCTAGATGGTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACAGGAC						
Al-Ots-B	GATGGTGTTTCTAGATGGTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACAGGAC						
Consensus	TGGATAAACAAGGCAGCAGAAACACTGCCACAGTACTGGGAGAGGAACACTGGGAATTNC						
Al-Ots-M	TGGGTAAACAAGGCAGCAGAAACACTGCCACAGTACTGGGAGAGGAACACTGGGAATTGC						
Al-Ots-H	TGGATGAACAAGGCAGCAGAAACACTGCCACAGTACTGGGAGAGGAACACTGGGAATTGC						
Al-Ots-D	TGGGTAAACAAGGCAGCAGAAACACTGCCACAGTACTGGGAGAGGAACACTGGGAATTGC						
Al-Ots-P	TGGATGAACAAGGCAGCAGAAACACTGCCACAGTACTGGGAGAGGAGAGACAGGGAATTGC						
Al-Ots-L	TGGATGAACAAGGCAGCAGAAACACTGCCACAGTACTGGGAGAGGAGAGACAGGGAATTGAC						
Al-Ots-T	TGGATGAACAAGGCAGCAGAAACACTGCCACAGTACTGGGAGAGGAACACTGGGAATTGC						
Al-Ots-N	TGGATAAACAAGGCAGCAGAAACACTGCCACAGTACTGGGAGAGGAGAGACAGGGAATTGC						
Al-Ots-E	TGGATAAACAAGGCAGCAGAAACACTGCCACAGTACTGGGAGAGGAACACTGGGAATTTC						

Al-Ots-C	TGGATAAACAAAGGCAGCAGAAACACTGCCACAGTACTGGGAGAGG <b>GAGACA</b> GGGA <b>TTTTC</b>
Al-Ots-I	TGG <b>G</b> TAAACAAGGCAGCAGAA--C-C--C-ACAGTACTGGGAGAGGAACACTGGGA <b>TTTTC</b>
Al-Ots-R	TGG <b>G</b> TAAACAAGGCAGCAGAA--C-C--C-ACAGTACTGGGAGAGGAACACTGGGA <b>TTTTC</b>
Al-Ots-A	TGGATAAACAAAGGCAGCAGAAACACTGCCACAGTACTGGGAGAG <b>TGAGACA</b> GGGA <b>TTTTC</b>
Al-Ots-B	TGGATAAACAAAGGCAGCAGAAACACTGCCACAGTACTGGGAGAG <b>TGAGACA</b> GGGA <b>TTTTC</b>
Consensus	AAGGGTGCCCAGCAGACTTTCAAAGCCAACATCGATATTGTAAAGCAG
Al-Ots-M	AAGGGTGCCCAGCAGACTTTCAAAGCCAACATCGATATTG <b>C</b> AAAGCAG
Al-Ots-H	AAGGGT <b>G</b> ACCAGCAGACTTTCAAAGCCAACATCGATATTGTAAAGCAG
Al-Ots-D	AAGGGT <b>G</b> ACCAGCAGACTTTCAAAGCCAACATCGATATTGTAAAGCAG
Al-Ots-P	AAGGGT <b>G</b> ACCAGCAGACTTTCAAAGCCAACATCGATATTG <b>C</b> AAAGCAG
Al-Ots-L	AAGGGTGCCCAGCAGACTTTCAAAGCCAACATCGATATTGTAAAGCAG
Al-Ots-T	AAGGGT <b>A</b> CCCAGCAGACTTTCAAAGCCAACATCGATATTGTAAAGCAG
Al-Ots-N	AAGGGT <b>A</b> CCCAGCAGACTTTCAAAGCCAACATCGATATTGTAAAGCAG
Al-Ots-E	AAGGGTGCCCAGCAGACTTTCAAAGCCAACATCGATATTG <b>C</b> AAAGCAG
Al-Ots-C	AAGGGT <b>G</b> ACCAGCAGACTTTCAAAGCCAACATCGATATTG <b>C</b> AAAGCAG
Al-Ots-I	AAGGGT <b>AA</b> ACCAGCAGACTTTCAAAGCCAACATCGATATTG <b>C</b> AAAGCAG
Al-Ots-R	AAGGGT <b>G</b> ACCAGCAGACTTTCAAAGCCAACATCGATATTG <b>C</b> AAAGCAG
Al-Ots-A	AAGGGTGCCCAGCAGACTTTCAAAGCCAACATCGATATTGTAAAGCAG
Al-Ots-B	AAGGGTGCCCAGCAGACTTTCAAAGCCAACATCGATATTGTAAAGCAG

## APPENDIX C

The nucleotide sequences of the 4 MH class II  $\beta_1$  alleles found in the study population of Chinook salmon from Chapter three. Nucleotide differences are in grey.

	1	10	20	30	40	50	60
Consensus	GGTATAGAGTTTATAGACTCTTATGTTTTCAATAAGGTTGAACATATCAGATTCAACAGC						
B1-Ots-A	GGTATAGAGTTTATAGACTCTTATGTTTTCAATAAGGCTGAATATATCAGATTCAACAGC						
B1-Ots-B	GGTATAGAGTTTATAGACTCTTATGTTTTCAATAAGGTTGAACATATCAGATTCAACAGC						
B1-Ots-C	GGTATAGAGTTTATAGACTCTTATGTTTTCAATAAGGTTGAAATATATCAGATTCAACAGC						
B1-Ots-D	GGTATAGAGTTTATACACTCTTATGTTTTCAATAAGGTTGAACATATCAGATTCAACAGC						
Consensus	ACTGTGGGGAGGTATGTTGGATACACTGAGCTGGGTNTGAAGAATGCAGAAGCATGGAAC						
B1-Ots-A	ACTGTGGGGAGGTATGTTGGATACACTGAGCTGGGTGTGAAGAATGCAGAAGCATGGAAC						
B1-Ots-B	ACTGTGGGGAGGTATGTTGGATACACTGAGCTGGGTCTGAAGAATGCAGAAGCATGGAAC						
B1-Ots-C	ACTGTGGGGAGGTATGTTGGATACACTGAGCTGGGTGTGAAGAATGCAGAAGCATGGAAC						
B1-Ots-D	ACTGTGGGGAGGTATGTTGGATACACTGAGCTGGGTCTGAAGAATGCAGAAGCATGGAAC						
Consensus	AAAGGTCCTCAGCTGGGTCAAGAGCAGGCGGAGCTGGAGCGTTTCTGTAAGCCTAACGCT						
B1-Ots-A	AAAGGTCCTCAGCTGGGTCAAGAGCAGGCGGAGCTGGAGCGTTTCTGTAAGCCTAACGCT						
B1-Ots-B	AAAGGTCCTCAGCTGGGTCAAGAGCAGGCGGAGCTGGAGCGTTTCTGTAAGCCTAACGCT						
B1-Ots-C	AAAGGTCCTCAGCTGGGTCAAGAGCAGGCGGAGCTGGAGCGTTTCTGTAAGCCTAACGCT						
B1-Ots-D	AAAGGTCCTCAGCTGGGTCAAGAGCAGGCGGAGCTGGAGCGTCTCTGTAAGCCTAACGCT						
Consensus	GCTCTCCACTACAGAGCCATACTGGACAAGA						
B1-Ots-A	GCTCTCCACTACAGAGCCATACTGGACAAGA						
B1-Ots-B	GCTCTCCACTACAGAGCCATACTGGACAAGA						
B1-Ots-C	GCTCTCCACTACAGAGCCATACTGGACAAGA						
B1-Ots-D	GCTCTCGAGTACAGAGCCATACTGGACAAGA						

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